MECHANISMS OF SMOKE-ELICITED SEED GERMINATION RESPONSES AND POSSIBLE CANDIDATE ACTIVE COMPOUNDS IN SMOKE

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ABSTRACT

Smoke derived from burning plants has been well recognized as a seed germination promoter in a variety of plant species in many ecosystems. Previous studies revealed that smoke from legumes resulted in different germination responses compared to that from graminoids. However, the mechanisms for such differences remain unclear. This research was conducted to understand how different smoke types interact with germination conditions to improve germination, to identify bioassay species among fire-ephemerals that respond positively to alfalfa smoke, and to identify germination stimulants in alfalfa smoke. Salad Bowl lettuce (*Lactuca sativa*) seeds treated with serial dilutions of smoke solutions from eight plant species and incubated at 25/25 °C or 25/15 °C in 12 h light/12 h darkness or 24 h darkness for three days. Seeds of six fire-ephemeral species from Africa and wild mustard (*Sinapis arvensis* L.) were primed in serial dilutions of alfalfa or/and wheat smoke solutions or distilled water for 24 h at 20 °C in darkness for one week and subsequently incubated at species-specific germination conditions. A lettuce seed bioassay, conducted at 25/15 °C in darkness, was used to identify active fractions and compounds derived from alfalfa smoke solution. Fractions were prepared by reversed phase separations (resin and C18 RP-CC), and compounds present were determined by HPLC, GC-MS and NMR and comparison of spectra with library spectra of known compounds. Lettuce seed germination percentage was improved by diluted smoke solutions from all plants at 25/15 °C in darkness, but by only two graminoid-based smoke types (wheat and *E. curvula*) at a constant 25 °C. Similarly, the germination rate of lettuce seeds treated with alfalfa or wheat smoke solution was enhanced mainly at 25/15 °C under darkness. Therefore, the efficiency of compounds in smoke solutions in replacing the effect of light appears to be greater at 25/15 °C than at 25 °C. Resin fractions, EtOAc separated neutral fraction, and C18 fractions of alfalfa smoke solution enhanced germination in a lettuce bioassay, but KAR1 was not present as a major germination stimulant in alfalfa smoke based on fractions. Phenolic compounds such as hydroquinone and catechol were more evident as possible active germination promoters in alfalfa smoke, to which six fire-ephemerals responded positively. Germination of wild mustard seeds increased with increasing smoke solution concentration. A bioassay based on mustard seed germination has the potential to guide the identification of active compounds isolated by fractionation from complex mixtures. Non-KAR1 active compounds in alfalfa smoke may have broad applicability in weed control in agroecosystems.

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I dedicate my thesis work to my parents, my teachers and my lovely wife Rasanwada who instilled in me the desire and love for studying plants.

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LIST OF ABBREVIATION

ABA	Abscisic acid ((2*Z*,4*E*)-5-[(1*S*)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl]-3-methylpenta-2,4-dienoic acid)
ANOVA	Analysis of variance
APT	Attached proton test
COSY	Correlation spectroscopy
d4-CD_3OD	deuterated methanol
EtOAc	Ethyl acetate
GA_3	Gibberellic acid ((1*R*,2*R*,5*S*,8*S*,9*S*,10*R*,11*S*,12*S*)-5,12-dihydroxy-11-methyl-6-methylidene-16-oxo-15-oxapentacyclo[9.3.2.15,8.01,10.02,8]heptadec-13-ene-9-carboxylic acid)
GC	Gas chromatography
GC-ESI-MS	Gas chromatography-electrospray ionization-mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
HPLC-DW	HPLC grade distilled water
HSQC	Heteronuclear single quantum coherence
ISTA	International seed testing association
KAR1	Karrikinolide
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
RP-CC	Reverse phase column chromatography
SK	Saskatchewan
SSSC	Saskatchewan Structural Science Center
U of S	University of Saskatchewan

CHAPTER 1. INTRODUCTION

Fire of natural or anthropogenic origin is a disturbance that drives succession and regeneration in ecosystems through direct or indirect effects (Bond & van Wilgen, 1996, 2012; Buhk & Hensen, 2006). Fire-associated smoke can break seed dormancy and/or promote germination in many plant taxa, and this remarkable effect occurs irrespective of plant life form, seed shape, and seed size (van Staden et al., 2000; Dixon et al., 2009). Other critical biological effects of smoke include stimulating seedling growth (Jain et al., 2006; Jain & van Staden, 2007; Moreira et al., 2010), seedling vigor (Sparg et al., 2005), and root initiation and hypocotyl development (Taylor & van Staden, 1996).

Ren & Bai (2016a) demonstrated that a highly concentrated smoke solution produced from a burnt herbaceous legume (alfalfa, *Medicago sativa* L.) promoted seed germination and seedling growth of fire-responsive *Artemisia frigida* and *Conyza canadensis.* They found no effect with a highly concentrated smoke solution produced from graminoids (Ren & Bai 2016a) and reported the absence of KAR1 in alfalfa smoke, thereby suggesting the presence of unidentified seed germination stimulant/s in smoke derived from alfalfa plant materials (Ren et al., 2017). Interestingly, alfalfa smoke solution did not stimulate lettuce seed germination (a common bioassay species), necessitating a search for alternative bioassay species.

Smoke contains thousands of chemical compounds, but only a few active germination stimulants have been isolated (Flematti et al., 2009, 2011b). The previously identified stimulant KAR1 (one of active compounds in smoke) does not promote germination in all species (Flematti et al., 2011a; Wang et al., 2017). Characterizing those active compounds has broad application such as grassland regeneration (e.g., Fescue Prairie), stimulating crops, medicinal plants, and rare, endangered, and threatened species, and weed control by stimulating dormant weed seed banks (Stevens et al., 2007). Known fire-ephemerals are more sensitive to smoke stimuli in germination (Tieu et al., 1999; Chiwocha et al., 2009).

Alfalfa smoke solution has been tested with fire-responsive species from Fescue Prairie, and a positive response in germination was found in seeds primed with different smoke concentrations (Ren & Bai, 2016a). In another study, seed germination of a weedy species belonging to the Poaceae family was improved by a smoke solution prepared by burning a leguminous plant, *Bauhinia variegate* (Kamran et al*.,* 2014). They found that the reduced ABA level and increased α-amylase activity as a sign for rising GA level during germination after smoke treatment.

The objectives of these experiments were: 1) to compare the ability of smoke derived from alfalfa, wheat and South African 'fire-ephemerals' to germinate lettuce seed (standard bioassay) under differing growth conditions; 2) to understand how and which fire-ephemeral species respond to smoke during germination; 3) to identify alternative novel bioassay species; and 4) to isolate and identify chemical compounds in alfalfa smoke that can enhance seed germination in the Salad Bowl lettuce bioassay. I hypothesized that 1) different smoke types stimulate seed germination responses differently; 2) temperature and light interact with smoke types to elicit different germination responses; 3) seed germination of fire-ephemeral species responds positively to alfalfa and/or wheat smoke solutions, and; 4) non-KAR1 chemical compounds in alfalfa smoke promote seed germination.

CHAPTER 2. LITERATURE REVIEW

2.1 Role of fire on seed germination

2.1.1 Plant regeneration and succession after fire

Fire regulates functional and structural properties of many plant communities through above-ground regeneration through vegetative reproduction (resprouters) and seedling recruitment from soil seed banks (seeders) (Keeley & Zedler, 1978; Sousa, 1984; Paula & Pausas, 2008). The adaptation of plants to fire is reflected in both physiological and morphological reproductive traits. Pausas & Keeley (2014) estimated that 57% of species from 139 families in Mediterranean ecosystems in North America and Europe have resprouted after fire events, which is typical for perennial dicotyledonous (Wells, 1969). Perennials can reproduce from vegetative propagation and seeds (Fenner & Thompson, 2005) and there can be trade-offs between the two (Paula & Pausas, 2008). Resources may be allocated differently for different reproductive strategies (Fenner & Thompson, 2005).

Fire is a prominent cause of secondary succession in many communities (Clements, 1916; Buhk & Hensen, 2006). Heat associated with fire promotes germination of seeders over resprouters (Paula & Pausas, 2008) as dormancy can be broken by high temperature (Moreira & Pausas, 2012). Outcomes of plant communities being burned, such as exposure to light, smoke, heat, the release of nutrients, and modifications on soil moisture conditions, as well as their interactions, affect germination success of seeders in plant communities (Van der Valk, 1992; Bazzaz, 1996).

Stored energy in plants is rapidly released during the combustion process. However, this depends on the combustion phases. Unlike in pre-ignition and flaming phases, smouldering (residual) phase releases little heat and large amount of smoke after consuming a little of plant materials (fuel) (Hao & Babbitt, 2007). It was successful to generate seed-germination promotive activity by smouldering plant material at 180 °C for 30 minutes (Flematti at al., 2015). The temperature ranges *ca.* 165 °C during plant material burning inside the bin (Abu, 2014).

2.1.2 Physical effects of fire on seed germination

Heat shock during a fire and changes in post-fire light quantity and quality are physical effects of a fire event (van Staden et al., 2000). Greater temperature fluctuations on or near the soil surface after fire help release seed dormancy and stimulate seed germination in many plant species

(Baskin & Baskin, 2014). The scarification of hard seed coats (physical dormancy) by heat promotes seed germination (Warcup, 1980; Baskin & Baskin, 1998). These physical processes are common among hard-seeded species belong to Fabaceae, Cistaceae, Ericaceae, Malvaceae, Pinaceae, Proteaceae, and Rhamnaceae families (Keeley & Fotheringham, 2000; Dixon & Barrett, 2003; Reyes & Trabaud, 2009; Moreira et al., 2010). In contrast, viable seeds in the uppermost soil layer or litter layer may be killed by flames or heat (Archibold et al., 1998).

The quality and quantity of light are modified by fire due to the reduction or removal of vegetation canopies and litter (Light, 2006; Ghebrehiwot, 2010). The red: far-red ratio increases after a fire, promoting seed germination (Silvertwon, 1980) and plant growth (Thompson & Harper, 1988).

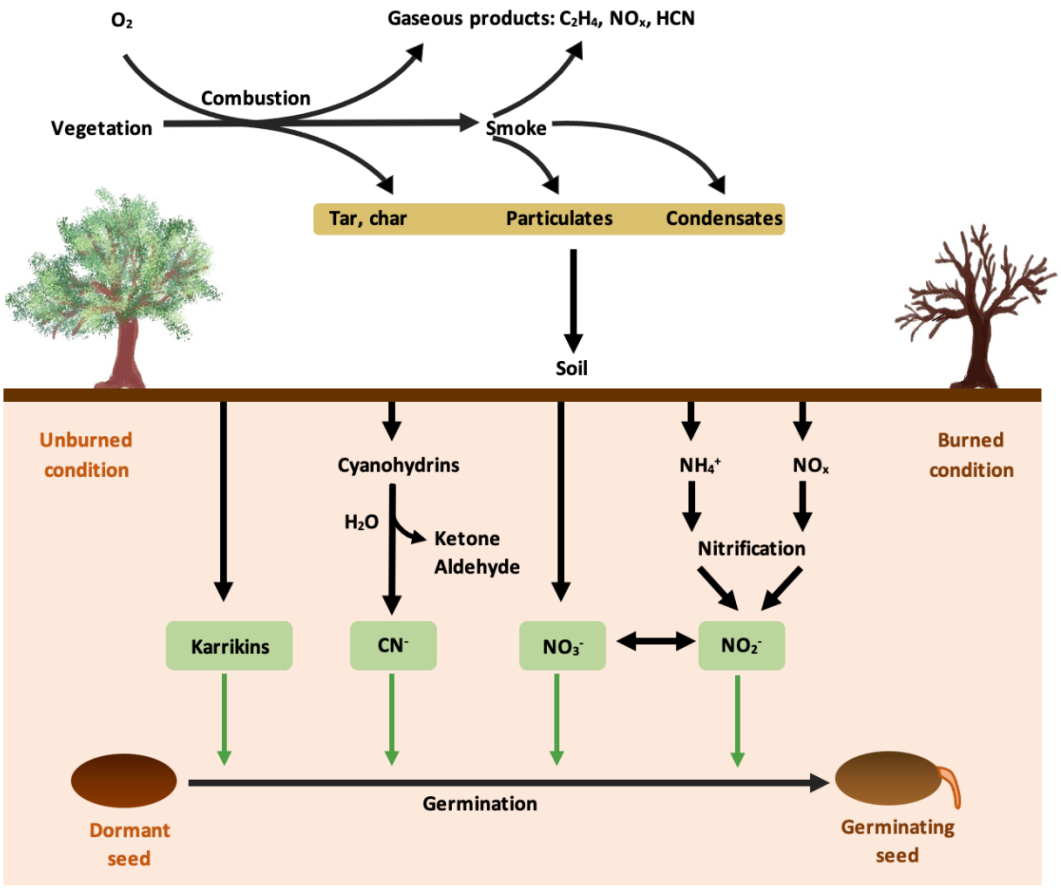


Figure 2.1 Fire-related cues responsible for breaking seed dormancy and promoting germination. Crude smoke, Karrikins, char/tar, CN^-, NO^-_3, and NO^-_2 elicit positive seed germination responses. NO_x includes NO or NO_2 derived during smoldering/slow burning of plant materials or soil microbiota activity. Microbial nitrification results in the oxidation of NH_4^+ or NO_x to NO_2^- (nitrite) or NO_3^- (nitrate). Chemicals infiltrate into soil layers with the rainwater (adapted and redrawn from Nelson et al., 2012).

2.1.3 Chemical effects of fire on seed germination

Burning of plant materials results in both volatile and non-volatile chemical cues (Figure 2.1) that influence seed dormancy release and germination (Ghebrehiwot, 2010). Smoke, ash, charred wood, ethylene, and nitrogenous compounds are the most cited chemical cues affecting seed dormancy and germination after a fire (Baskin et al., 2003; Light, 2006). Seed germination of the Californian Chaparral annual *Emmenanthe penduliflora* was promoted after keeping seeds near charred stems of *Adenostoma fasciculatum* (Wicklow, 1977). Ren & Bai (2016b) reported the

positive effects of both direct and interactive effects of ash and smoke on richness, density, and emergence rate of seedlings from the soil seed bank in Fescue Prairie. However, charred wood and ash treatments may not always significantly affect seed germination, as demonstrated in a study of 21 Mediterranean species (Buhk & Hensen, 2006).

Ethylene acts as an indirect signal related to the ability to sense ambient water availability (Baskin et al., 2003), and this volatile compound stimulates seed germination in selected species (KeÇpczyński & KeÇpczyńska, 1997; Baskin & Baskin, 1998). However, it was not the active compound in smoke solution which stimulated seed germination of Grand Rapids lettuce seeds (Jäger et al., 1996b). Nitrogenous compounds, mainly nitrites (NO_x to NO_2^-) and nitrates (NO_3^-), can break seed dormancy, promoting germination and seedling emergence (Henig-Sever et al., 2000; Nelson et al., 2012). Nitrates (NO_3^-) enhance the germination of dormant seeds in a consistent pattern (Keeley & Fotheringham, 1997). California Chaparral annual *Emmenanthe penduliflora* responded positively to nitrogenous compounds in smoke (Keeley & Fotheringham, 1997, 1998). Several other Chaparral (Keeley & Fotheringham, 1998) and Western Australian (Bell et al., 1999) species have shown similar promotive effects, while Mediterranean species (Luna & Moreno, 2009) showed a neutral effect after KNO_3 treatment. Light et al. (2003) suggested that nitrogenous oxides (NO) are not likely responsible for smoke-elicited lettuce seed germination. Furthermore, ammonium and ethyne compounds were excluded as active compounds in smoke solutions on stimulating effects of lettuce seed germination (van Staden et al., 1995b; Jäger et al., 1996b).

2.2 Smoke as a seed dormancy breaker and germination cue

Seed dormancy is an intrinsic property of seeds that prevents them from germinating under conditions otherwise favorable for germination (Baskin & Baskin, 1998). After imbibition, dormant seeds may respond to external stimuli, including alternating temperatures, cooling, light, and various chemical cues (Bewley, 1997). Smoke, as a potent inducer of seed germination, was prominent among other fire-related cues (De Lange & Boucher, 1990; Brown, 1993b). A landmark study demonstrated enhanced seed germination of a South African species, *Audouinia capitata* (Bruniaceae), after passing gaseous smoke above a soil tray containing its seeds (De Lange & Boucher, 1990). Smoke, and its active stimulants, is capable of breaking seed dormancy and promoting germination in many plant taxa, including Restionaceae (Brown et al., 1994), Asteraceae, Ericaceae, Proteaceae (Brown, 1993b; Brown et al., 1993), Mesembryanthemaceae (Pierce et al.,

1995) and Poaceae (Baxter et al., 1995). Smoke action to break seed dormancy and stimulate germination relies on whether the general habitat is fire-prone or fire-free (Tieu et al., 1999; van Staden et al., 2000; Chiwocha et al., 2009). Plant-derived smoke affects plant regeneration in diverse ecosystems such as the Western Cape fynbos (Brown et al., 2003), Californian Chaparral (Keeley & Fotheringham, 1998), Australian kwongan (Dixon et al., 1995; Roche et al., 1997), Mediterranean Basin (Crosti et al., 2006) and Canadian Fescue Prairie (Abu et al., 2016; Ren & Bai, 2016b, 2017). Smoke promoted seed germination in several Canadian Fescue Prairie and Western Australian species under both field and greenhouse conditions (Dixon et al., 1995; Ren & Bai, 2016b). Over 1200 species respond positively to plant-derived smoke by promoting seed germination (Dixon et al., 2009). This response is independent of seed size, shape, and plants' life form (Dixon et al., 1995).

Smoke-treated seeds germinate and grow into seedlings under a broader range of environmental conditions than non-treated seeds suggesting that smoke modifies physiological processes during germination and growth. For instance, tomato (*Lycopersicon esculentum*) seeds germinated at a wide range (between 10 ℃ and 40 ℃) after being primed with a bioactive compound in smoke (KAR1) (Jain et al., 2006). Smoke helps overcome inhibiting environmental conditions such as water stress (Ghebrehiwot et al., 2008; Thomas et al., 2010) and high-temperature stress (Ghebrehiwot et al., 2008) during seed germination and seedling growth. An isolated active stimulant in smoke, butenolide (3-methyl-2H-furo[2,3-c]pyran-2-one), has been shown to promote seed germination and seedling vigor in a broad range of plant species (van Staden et al., 2006; Kulkarni et al., 2007). After exposing tomato seeds to smoke solutions, root length increased by ten times (Jain et al., 2006). Selected species from the Mediterranean Basin and South Africa increased seedling growth after smoke solution treatment (Sparg et al., 2006; Moreira et al., 2010). Smoke enhanced species richness and seedling densities in South African mesic grasslands (Ghebrehiwot et al., 2012). In addition, plant-derived smoke can initiate other significant biological responses in plants, break dormancy in bulbs (Imanishi, 1983), elicit flowering, root initiation, and hypocotyl development (Taylor & van Staden, 1996), improve seedling vigor (Baxter & van Staden, 1994; Sparg et al., 2005) and promote somatic embryogenesis in the plants (Light et al., 2009).

2.2.1 Factors affecting smoke action on seed dormancy breaking and germination

2.2.1.1 Seed germination conditions

Smoke, and/or chemical compounds present in smoke, interact with various factors, including seed physiology and environmental conditions in breaking seed dormancy and subsequent germination. In general, seed dormancy breaking, and germination is the result of a combination of environmental factors (Benech-Arnold et al., 2000) such as light (Baskin & Baskin, 1998; Maloof et al., 2000), chilling, daily fluctuation in soil temperature (Pierce & Moll, 1994), high temperature (Standifer & Wilson, 1988), and low temperature (Baskin & Baskin, 1998). Hence, plant-derived smoke interacts with environmental factors like fire-origin heat (Thomas et al., 2003; Clarke & French, 2005), light regime (Brown & van Staden, 1997), incubating temperature (Brown et al., 1994; van Staden et al., 1995b; Ghebrehiwot et al., 2009) and soil nutrients (Ghebrehiwot, 2010; Kulkarni et al., 2012) to break seed dormancy and promote seed germination. The combined effect of heat and smoke on seed germination stimulation has been widely reported (Morris, 2000; Thomas et al., 2003; Keeley et al., 2005; Clarke & French, 2005). Seed germination of an endangered Australian shrub, *Epacris stuartii* Stapf. (Keith, 1997) and seven *Grevillea* species (Morris, 2000) were stimulated by the additive effect of smoke and heat but not by heat or smoke alone.

The positively photoblastic *L. sativa* seeds require light to germinate. However, under darkness, germination occurs in *L. sativa* seeds that treated with aqueous smoke solution or KAR1 (an active compound in smoke) indicating that aqueous smoke solution or KAR1 can substitute the light requirement for the seed germination (Drewes et al., 1995; Thomas & van Staden, 1995). Similarly, after smoke treatment, negatively photoblastic seed germination of *Syncarpha vestita* was reported in the presence of light (Brown, 1993a; Brown & van Staden, 1997). Temperature-dependent seed germination stimulated by smoke was reported in several studies. Ghebrehiwot et al. (2009) found that increasing temperatures enhance the positive effects of smoke solutions on seed germination and seedling growth of *Hyparrhenia hirta* L. (thatching grass), *Panicum maximum* (Guinea grass), and *Aristida junciformis* (bristle grass). In another study, seeds of several Canadian forages primed in smoke solutions had higher percent seed germination under contrasting temperature regimes (Abu et al., 2016). After a fire, changes in micro-environmental conditions may improve (Nelson et al., 2012) or worsen (Turner et al., 1997) nutrient availability for newly emerged seedlings. Seed dormancy status (Nelson et al., 2012), different seed lots and harvest years (Long et

al., 2011), dry after-ripening (Adkins & Peters, 2001), and soil burial (Tieu et al., 2001) all affect the promotive effect of smoke on seed germination.

2.2.1.2 Smoke solution-related factors

Smoke-mediated seed germination and seedling growth depend on the concentration or dose of the smoke solution. In general, a high concentration smoke solution inhibits or has a neutral effect on germination (Abu et al., 2016; Ren & Bai, 2016a), whereas diluted smoke solutions mostly promote seed germination (van Staden et al., 1995a; Brown & van Staden, 1997). Crude smoke solution diluted from 100 to 1000-fold enhanced seed germination of *L. sativa* while the concentrated smoke dilutions (i.e., 1/1, 1/10 v/v) inhibited or reduced seed germination (Drewes et al., 1995). In contrast, concentrated smoke solution promoted seed germination of *Conyza canadensis* (Ren & Bai, 2016a), *Aristolochia debilis* Siebold & Zucc. (Zhou et al., 2014) and *S. arvensis* (Godakanda et al., 2020). Daws et al. (2008) reported that the smoke-isolated active butenolide, 3-methyl-2H-furo [2,3-c]-pyran-2-one (KAR1) solution and burnt cellulose solution (Flematti et al., 2004) promoted seed germination of several species at high concentrations. The presence of seed germination inhibiting compounds in crude smoke solution compared to isolated butenolide solution may be the reason for the negative or neutral effects of smoke on seed germination percentage, rate, and seedling growth (Daws et al., 2007). The active stimulant in smoke, KAR1, does not inhibit seed germination at high concentrations (van Staden et al., 2004) while retaining its effectiveness even at 10^{-9} M. Seed germination that is sensitive to smoke concentration further suggests that possible inhibitors are present in smoke solutions that can be neutralized by dilution (Drewes et al., 1995; Brown & van Staden, 1997). A novel germination inhibitory compound, trimethylbutenolide [TMB; 3,4,5-trimethylfuran-2(5H)-one], has been isolated from smoke water (Light et al., 2010). It was shown to have an inhibitory effect on *L. sativa* seed germination when applied together with the known promotive compound, KAR1 (Light et al., 2010). It is important to note that the sensitivity to stimulants or/and toxic compounds in smoke varies among plant species (Jäger et al., 1996a; Adkins & Peters, 2001; Ren & Bai, 2016a).

2.3 Smoke-derived active stimulants

2.3.1 Bioassay-guided chemical isolations from smoke solutions

Researchers have attempted to isolate active compounds in smoke water that stimulate seed germination using a variety of chemical separation and detection methods (Light, 2006; Flematti et al., 2008). Analysis of a chemically complex mixture such as smoke solution is a difficult task. To minimize the challenge, Flematti et al (2008a) used a bioassay-guided fractionation process to evaluate fractions after chemical separation processes. Drewes et al. (1995) made the breakthrough discovery that light-sensitive lettuce (*L. sativa*) seeds germinate quickly when exposed to smoke water under fully darkness condition. Since then, this species has been used as the bioassay species in smoke water isolation studies. However, different plant species have been evaluated to research smoke-stimulated seed germination as well as different active compounds in smoke. *Nicotiana attenuate* seeds were used to evaluate the efficacy of fractions of smoke extracts and pure chemicals to stimulate seed germination (Baldwin et al., 1994). *Lysinema ciliatum* (Roche et al., 1997) and *Stylidium affine* (Tieu et al., 1999) seeds were used as bioassay species to test the effects of smoke solutions on seed germination.

In most studies, chemical compound extraction and isolation from complex smoke water solutions were conducted with the classic liquid-liquid partitioning process or solvent extraction methods (Flematti et al., 2004; van Staden et al., 2004; Wang et al., 2017; Kamran et al., 2017; Ren et al., 2017). Conventional natural product chemistry provides an initial point of separation of highly complex smoke water solutions into an aqueous portion (insoluble in organic solvents), organic portion, weak acids (NaOH-soluble), and neutral fractions (neither acid nor base soluble). Separation is achieved through liquid-liquid partition using a suitable, predetermined organic solvent. It has been hypothesized that active compounds might go to one partition based on the polarity of their molecules while eliminating unwanted effects of a complex chemical matrix of smoke water. The salad bowl lettuce bioassay can then be used to select the active portions. Flematti et al. (2008) pioneered modern chromatographic methods such as C18 reversed phase column chromatography (RP-CC) as a replacement for alumina column chromatography to recover more materials from smoke water solutions.

During previous isolation and detection attempts to isolate active compounds in plant-derived smoke, gas chromatography (GC) and/or high-performance liquid chromatography (HPLC) were consistently used (van Staden et al., 2004; Flematti et al., 2004, 2011a). In modern spectroscopic

detection methods, chromatographic separation techniques are coupled with diode array detectors (DAD) and/or mass spectrometry (MS) to increase the resolution of separation and detection power. Methods based on gas chromatography-mass spectrometry (GC-MS) and HPLC-DAD-MS have been routinely adopted in smoke compound identification (Light, 2006; Flematti et al., 2008; Wang et al., 2017). Higher sensitivity and selectivity, and its ability to detect structural parameters of the analytes make mass spectrometry (MS) an outstanding tool in plant metabolite detection studies compared to other detection methods. GC-MS and atomic absorption (AA) instruments successfully identified 71 compounds in active smoke fractions using *Nicotiana attenuata* seeds as a bioassay species (Baldwin et al., 1994). The National Institute of Standards and Technology (NIST) harbors 'mass spectral library' with over 2 million mass spectral fingerprints measured for over 350,000 chemical compounds (William, 2019). Comparisons between the experimentally obtained mass spectra of unknown compounds and this library make it possible to identify possible chemical species quickly and accurately (Simon-Manso et al., 2013; William, 2019). Laborious sample preparation, missing of poor volatile active compounds and heat sensitivity are some of the drawbacks in GC methods.

Jäger et al. (1996b) studied *Themeda triandra* smoke extracts using chromatographic separation techniques such as thin-layer chromatography (TLC), semi-preparative HPLC, and analytical HPLC with a lettuce bioassay based on the cultivar Grand Rapids. The HPLC method is helpful to separate, monitor and quantify analytes in complex biological materials (Unger et al., 2010). Separation of a wide range of chemical compounds using HPLC can be achieved in combination with both mobile (solvent) and stationary (column) phases (Unger et al., 2013). HPLC is accompanied by different detectors, including ultraviolet (UV), ultraviolet-visible (UV/vis) and refractive (RI) detectors. Diode-array detectors (DAD) function by comparing the retention times and UV spectra of an unknown analyte. DAD can detect multiple wavelengths simultaneously in the 185 to 700 nm range (Dong, 2019).

2.3.2 Active chemicals isolated from smoke

Prior to any chemical isolation studies, several known compounds with positive effects promoting seed germination were identified as occurring in plant-derived smoke. However, those known chemical compounds such as carbon dioxide, ethylene, methane, nitrates were eliminated as likely active chemical compounds in smoke solutions (Keeley & Fotheringham, 1998). It is well

known that ethylene acts as a seed germination stimulant in both dormant and non-dormant seeds (Matilla & Matilla-Vazquez, 2008; Corbineau et al., 2014). Nevertheless, ethylene alone could not break the dormancy of wild oat (*Avena fatua*) caryopses. However, the combination of ethylene and smoke-derived KAR1 (bioactive in smoke) break seed dormancy, indicating that KAR1-mediated dormancy breaking needs some level of endogenous ethylene in seeds (Kępczyński & van Staden, 2012). Preston et al. (2004) reported that except in raw smoke solution, nitrous oxide (NO_x) alone does not stimulate seed germination in smoke responsive species. There was a significant difference in *Nicotiana attenuata* seed germination after being treated with nitrogenous compounds and pyrolysis products (smoke) of α-cellulose (Baldwin et al., 1994), indicating the possible absence of any NO_2 or NO nitrogenous compounds in α-cellulose and hemicellulose. However, after finding cyanide as a natural seed germination promoter in smoke (Flematti et al., 2011a), re-evaluation of the nitrogen oxides in smoke was suggested (Nelson et al., 2012). If present in smoke, oxidizing agents such as hydrogen peroxide may promote seed germination through increased ethylene and gibberellic acid (GA) biosynthesis pathways (Liu et al., 2010b; Barba-Espín et al., 2012). Acidic components, such as sulphuric acid, present in smoke may possibly act as chemical scarification agents. These have been shown to alter seed coat permeability and, thereby, break seed coat-imposed dormancy in *Emmenanthe penduliflora* seeds primed in smoke solution (Keeley & Fotheringham, 1997; Egerton-Warburton, 1998). After the fire in Chaparral ecosystems, seed germination in annuals was promoted by acidic components from smoke (Keeley & Fotheringham, 1998).

While the search for and isolation of germination stimulants are ongoing, different active compounds have been isolated from plant-derived smoke solutions that break seed dormancy and promote germination. Butenolide, 3-methyl-2H-furo [2,3-c]-pyran-2-one, was a highly active germination stimulant first isolated from both plant-derived smoke (van Staden et al., 2004) and burnt cellulose (Flematti et al., 2004). This butenolide was renamed as karrikinolide (KAR1), where "karrik" means the smoke in a language native to Australia (Commander et al., 2008; Flematti et al., 2009). In a structure-activity study of karrikin, Nelson et al. (2012) demonstrated that to stimulate seed germination, the presence of methyl group at the 3rd carbon of the cyclic structure is essential. Furthermore, the addition of methyl groups into C-4 or C-7 locations minimizes the germination bioactivity of this molecule. Major KAR1 and additional five karrikin analogs (KAR_2-KAR_6) were proposed and confirmed by chemical synthesis (Flematti et al., 2009). Among these, KAR1 has the highest concentration among all karrikin analogs and was therefore considered the main chemical species in plant-derived smoke responsible for breaking seed dormancy, promoting germination

percentage and rate, and seedling growth. The cellulose was identified as the major and fundamental molecule in plants that leads to the production of bioactive KAR1 during natural fire (Flematti et al., 2011b). In addition, heating sugars with protein or amino acids was also reported as a possible route of formation of KAR1 (Light et al., 2005). Keeley & Pizzorno (1986) demonstrated that heated xylan and heated glucuronic acid as seed germination stimulants which are important constituents of wood hemicelluloses.

The stimulating effects of KAR1 on seed germination and/or seedling vigor have been reported in both fire-prone habitats (Flematti et al., 2004; Merritt et al., 2006) and fire-free environments (Kępczyński, 2018) in a variety of plant communities. This includes crops such as lettuce, tomato, okra, bean, maize, and rice (Kulkarni et al., 2006; van Staden et al., 2006; Jain et al., 2008; Kulkarni et al., 2011), weeds and hemi- and holo-parasitic seeds (Daws et al., 2007; Stevens et al., 2007), several Australian Asteraceae species (Merritt et al., 2006), and medicinal plants (Kulkarni et al., 2007). KAR1 was shown to be neither genotoxic nor toxic at $3 \times 10^{-10} – 10^{-4}$ M, suggesting its safe use on animal and human subjects (Light et al., 2009). Unlike crude smoke solution, KAR1 is not toxic or inhibitory at higher concentrations (van Staden et al., 2004). More importantly, KAR1 helps overcome environmental stress from water and temperature extremes, widening the environmental window for seed germination (Light et al., 2009). Tomato (*Lycopersicon esculentum*) seeds primed with KAR1 germinated at supra and sub-optimal temperatures (Jain et al., 2006). Some karrikin analogs, such as KAR2, were most effective in germinating *Arabidopsis* seeds (Nelson et al., 2009), suggesting a species-specific response towards karrikin analogs.

Karrikin (KAR1) was thought to be the major active stimulant in smoke (Nelson et al., 2012). However, in several studies, germination in seeds of some plant species was insensitive to KAR1 or nitrate but sensitive to smoke water. For instance, *Tersonia cyathiflora* (Gyrostemonaceae) and *Anigozanthos manglesii* (red and green kangaroo paw; Haemodoraceae) seeds were stimulated by smoke but not to KAR1 or nitrate (Dixon et al., 1995; Downes et al., 2010, 2014). This suggests that a non-karrikin stimulant in smoke promotes seed germination (Nelson et al., 2012) and led to the isolation of a second active compound in smoke, glyceronitrile, which releases cyanide after hydrolysis as the seed germination stimulant (Dziewanowska et al., 1979; Flematti et al., 2011a). Other than *A. manglesii*, 11 other smoke-responsive species germinated after being treated with a synthetic glyceronitrile (Flematti et al., 2011a). In their study, glyceronitrile was isolated in smoke produced by the combustion of agricultural feed straw (oaten hay).

The third active compound in smoke, catechol (1,2-benzenediol), was identified as a promoter of radicle and root hair elongation in *Nicotiana attenuata* Torr. (Wang et al., 2017). Catechol was isolated from both burnt vegetation and soil of the native habitat of *N. attenuata* in Arizona, USA. Previously, it was reported that catechol is very abundant in smoke (Baldwin et al., 1994; Montazeri et al., 2013). Similarly, the fourth active compound, hydroquinone (1,4-benzenediol), was isolated from smoke produced by smoldering leaves of the dioecious gymnosperm, *Ginkgo biloba* L. (Kamran et al., 2017). Hydroquinone at low concentration increased seed germination and seedling growth of lettuce seeds but was inhibitive at high concentration (Kamran et al., 2017). Hydroquinone was previously reported as a seed germination promoter in peanuts (Elwakil, 2003) and as a seedling growth agent in wheat (Li et al., 2009).

Considering the chemical structures and former findings (Albizati & Tracewell, 2012; NCBI, 2021a, 2021b), lignin in plants could be the starting material of hydroquinone and catechol which result after plant material burnings. Importantly, lignin is a component of plant cell wall and the second most abundant organic biopolymer after cellulose in plants, accounting *ca.* 30% of the organic carbon in the ecosphere (Boerjan et al. 2003). Albizati & Tracewell (2012) reported the production of hydroquinone and catechol from lignin under oxidative and higher temperature conditions. During the smouldering process of plant materials, a reduced atmospheric pressure and oxidative conditions are maintained inside the burning apparatus. These conditions may facilitate the above-mentioned reactions to produce seed germination stimulants, hydroquinone and catechol during plant material burning. In contrast, seed germination was not promoted by either heated or non-heated products of naturally occurring pure lignin (acetosyringone, ferulic acid, syringic acid, vanillyl alcohol) (Keeley & Pizzorno, 1986). This could be due to less chemical stability of burnt lignin products.

2.3.3 How long do seed germination stimulants remain in the soil?

Smoke solutions have retained their seed germination promotive ability after autoclaving (van Staden et al., 2000) and long periods of storage (at 10 °C) (Kulkarni et al., 2011). The selected karrikin standards are sufficiently stable during analytical conditions such as weakly acidic (pH 5.0) and neutral conditions (pH 7.0) and at temperatures of + 4 and + 22 °C (Hrdlička et al., 2019). This karrikinolide is water-soluble and stable at high temperatures (melting point: 118° to 119°C) (Flematti et al., 2004). However, further studies found that karrikins are not stable at higher

temperatures (Scaffidi et al., 2012b). A former study suggested that after a fire, seed germination stimulants can remain in the soil for over seven years (Preston & Baldwin, 1999).

In contrast, Scaffidi et al. (2012b) found that karrikins are unstable upon exposure to ultraviolet light, suggesting their likely decay on the soil under natural sunlight. Other aromatic compounds present in the smoke can act as natural 'sunscreens' to protect karrikins. Also, the quick infiltration of karrikins into the soil may increase its concentration and activity. Upon ultra-violet (UV) irradiation with a solar light source, the karrikin formed two novel head-to-head cage photodimers within the first hour and reached a maximum of around nine hours. Further irradiation degraded these photodimers (Scaffidi et al., 2012b). However, the presence of karrikins and not detecting those photodimers in the same soil samples collected several months after a fire indicated that only solid-state karrikin undergoes the dimerization (Scaffidi et al., 2012b). Also, other numerous chemical compounds present in smoke may protect karrikins from UV irradiation. This highlights the necessity of formulating suitable UV stabilizers with karrikins for effective use in commercial applications.

Another germination stimulant, hydroquinone, has chromophores which absorb light at wavelengths >290 nm, indicating the susceptibility to direct photolysis by natural sunlight. Non-ionized catechol does not absorb light >300 nm and, therefore, will not undergo photolysis. Specifically, vapor-phase hydroquinone and catechol will be degraded through photochemically-produced hydroxyl radicals (Gad & Pham, 2014). These reactions have a half-life in the air *ca.* 17 hours and *ca.* 2.8 hours, respectively. Hydroquinone in the particulate-phase will be dragged away from the atmosphere through wet and dry deposition (NCBI, 2021a,2021b). In general, hydroquinone and catechol do not undergo hydrolysis due to the lack of functional groups. Also, volatilization of these compounds occurs from moist soil surfaces, not from dry soil surfaces. (NCBI, 2021a,2021b)

2.3.4 Mechanism of smoke-stimulated seed germination

Although inconclusive, several pathways potentially explain the mechanisms of smoke-stimulated breaking of seed dormancy and promotion of germination. Smoke, as a chemical scarification agent, is a mechanism by which the seed coat cuticle is mechanically collapsed or damaged, thereby increasing the permeability to oxygen and water (Keeley & Fotheringham, 1997; Egerton-Warburton, 1998; Light, 2006). However, this mechanism may be species-specific (Baxter

et al., 1995; Keeley & Fotheringham, 1997). For instance, an obligate fire follower, *Emmenanthe penduliflora* (Wicklow, 1977; Christensen & Muller, 1975), produces seeds with physical dormancy (PY) (Baskin & Baskin, 1998). After treatment with smoke solution, water permeability of the sub-testa cuticle of *Emmenanthe penduliflora* seeds were changed and seed dormancy was broken (Egerton-Warburton, 1998). Morris (2000) demonstrated that germination of some Australian *Grevillea* species was stimulated significantly after smoke exposure alone as opposed to treatments such as heat or scarification. This indicates that smoke may act differently than chemical scarification agents.

Smoke may promote seed germination through a signal-mediated pathway (Baldwin et al., 1994) or by altering the sensitivity of seeds to phytohormones (Schwachtje & Baldwin, 2004; Nelson et al., 2009). Major phytohormones such as GA and ABA control seed dormancy and germination process in most species (Koornneef et al., 2002; Olszewski et al., 2002). Dormancy release and germination are governed by GA, while ABA extends dormancy and inhibits seed germination (Kucera et al., 2005; Finkelstein et al., 2008). Aqueous smoke solution and/or active compounds in smoke (KAR1) interacts with known plant hormones such as cytokinins, gibberellins, abscisic acid, and ethylene to promote seed germination and seedling growth. This suggests that smoke, or its active compounds, has hormone-like activity (Senaratna et al., 1999; van Staden et al., 2000; Gardner et al., 2001; Nelson et al., 2009). Depending on species, smoke-stimulated seed germination may occur because of changes in metabolism/synthesis of endogenous hormones and/or higher sensitivity to hormones (van Staden et al., 2000). KAR1 increased gibberellin sensitivity rather than changing endogenous ABA, GA1, GA3, and GA4 levels before the germination of *Stylidium maritium* Lowrie. (Chiwocha et al., 2009). However, in *L. sativa* and *Nicotiana attenuate* seeds, active compounds or smoke increased GA and decreased ABA endogenously (Gardner et al., 2001; Schwachtje & Baldwin, 2004). To explore cellular/molecular mechanisms, expressions of two genes (GA3ox1 and GA3ox2) responsible for GA biosynthesis were induced by KAR1 in dormant *Arabidopsis* seeds (Nelson et al., 2009). Karrikinolide (KAR1) has a butenolide fused to a pyran ring. Specifically, this butenolide ring is structurally in common with the novel plant hormones, strigolactones (GR24), that induce several physiological responses in plants. The hydrolysis of this butenolide ring is the proposed mode of action for Karrikinolide and GR24 after chemical structure-activity studies (Scaffidi et al., 2012a).

2.4 Germination characteristics

Eragrostis curvula (Schrad.) Nees (weeping lovegrass) is abundant in grasslands and indigenous to most parts of southern Africa where wildfires are frequent (Fynn & Naiken, 2009). *E. curvula* produces a prolific number of tiny seeds (12 000 seeds/plant) and colonizes fertile soils (Kirkman & Morris, 2003). Seeds of *E. curvula* prefer high temperature for germination (Maze et al., 1993); 2% and 96% at temperatures of 15 °C and 30 °C, respectively (Martin & Cox, 1984). The optimal range of temperatures for *E. curvula* seed germination is between 10.8 °C and 38 °C (Jordon & Haferkamp, 1989; Wester et al., 1986). Seed germination is not different in light and dark conditions (Maze et al., 1993). *Eragrostis curvula* seed germination decreases as the water potential in its soil or filter paper decreases (Wright et al., 1978; Maze et al., 1993). Seeds germinate better on the soil surface, and thick litter or burial (> 5 cm) inhibits seedling emergence (Cox & Martin, 1984; Dalrymple, 1970).

Cymbopogon pospischilii (K.Schum.) C.E.Hubb. produces fewer than 200 seeds per plant (Jones, 1968). Seed viability is low with varied seed production among plants (Robinson & Potts, 1950). Seeds can germinate over a wide range of diurnal temperatures (5 – 45 °C), light regimes, and under marginal water stress conditions (–0.55 MPa). More than 80% of seeds germinate within 12 hours at 30/20 °C with a 12 h photoperiod. Although the presence of light enhances seed germination slightly, seeds can germinate readily in darkness. The length of photoperiod does not influence germination (Chejara et al., 2008).

Indigofera hilaris Eckl. & Zeyh. seeds germinate well after fire (Martin, 1966). Germination studies are rare for this species, but another species of this same genus, *I. senegalensis*, showed high germination rates between 30 and 35 °C as well as breaking of physical dormancy (PY) after immersion in concentrated sulphuric acid (Sy et al., 2001).

Melinis nerviglumis (Franch.) Zizka. is a prolific seed producer. Germination was very low (4%) under a constant light regime at 30 °C (Ellis, 2010). Seeds germinate between 7 and 14 days at 21–24 °C and reached 50% at 21 °C under 12 h light/darkness (Liu et al., 2008). A similar species, *Melinis ripens* (natal grass), was not sensitive to light during germination. Higher germination (89%) was observed at 30 °C (Stokes et al., 2011).

Seeds in the Asteraceae family usually require light to break dormancy and for germination (Merritt et al., 2006; Karlsson & Milberg, 2007). Alternating temperature regimes promote germination (Wood et al., 2005; Honda & Katoh, 2007). *Helichrysum nudifolium* (L.) Less. is a sun-

loving and fast-growing perennial herb. *Helichrysum spp.* (everlasting) propagates extensively by seed and 20 °C and darkness is ideal for seed germination of this species (Piotto & Noi, 2003). Other similar species, such as *Helichrysum aureonitens* germinated best at 25 – 30 °C (Afolayan et al., 1997). GA_3 treatment significantly increased seed germination under continuous light, indicating the presence of physiological dormancy (Afolayan et al., 1997). *Vernonia capensis* (Houtt.) Druce. is a synonym of *Hilliardiella capensis* (Houtt.) H.Rob., Skvarla & V.A.Funk (Foden & Potter, 2005). Germination studies are rare for *Vernonia capensis* or *H. capensis* species. However, there is more information about other similar species, such as *Vernonia galamensis*, *Vernonia cinerascens*, a*nd Vernonia cinerea*. Seed germination of *Vernonia galamensis* was higher in light than dark at alternating temperatures (Nyamongo et al., 2009). Similarly, seed germination in both *Vernonia cinerascens* and *Vernonia cinerea* was higher in light than dark at high temperature (Shaukat et al., 2004).

High temperatures ≥28 °C inhibit lettuce seed germination. But this high temperature-induced thermoinhibition was reversed or overcome by the application of ABA biosynthesis inhibitor, fluridone (Gonai et al., 2004). In general, lettuce seeds require light to break dormancy and germination (Geneve, 1998). Photodormancy or skotodormancy can be induced in lettuce seeds in darkness at temperatures unsuitable for seed germination (Borthwick et al., 1952; Bewley, 1980). Up-regulation of genes such as LsGA3ox1 and LsGA3ox2 was observed after exposing lettuce seeds to red light. These genes are responsible for increasing seed GA content and thereby stimulating germination (Sawada et al., 2008). Similar to red light, smoke water solution promoted seed germination in darkness (van Staden et al., 1995c). Red light or smoke solution in darkness is responsible for converting chromoproteins from Pr form into Pfr, and this conversion helps to alleviate lettuce seed dormancy and germination (Drewes et al., 1995; Light, 2006; Seo et al., 2009). Nitric oxide can also enhance lettuce seed germination in darkness (Beligni & Lamattina, 2000).

In general, many weedy species require light and/or alternating temperatures to break dormancy (Baskin & Baskin, 1998; Daws et al., 2002; Batlla & Benech-Arnold, 2010). *Sinapis arvensis* L. (wild mustard) is a prolific seed producer (Warwick et al., 2000) and has little or no germination at constant temperatures between 5 and 30 °C in darkness (Goudey et al., 1987). Ten minutes of light exposure during incubation at 20 °C was identified as the optimum germination condition (Goudey et al., 1987). In a greenhouse study, Goudey et al. (1987) found that a combined KNO_3 and NH_4Cl treatment promoted seed germination to over 90% at 20 °C under a light. In contrast, nitrogen treatment inhibited germination rate and had a neutral effect on final germination

percentage (Luzuriaga et al., 2006). Germination was inhibited after irradiation with far-red light of dry *S. arvensis* seeds which was then overcome by red light irradiation, indicating that dry seed's light sensitivity occurs through the meta-Fa phytochrome pathway (Bartley & Frankland, 1984, 1985). Chemical scarification with concentrated H_2SO_4 was the fastest way of breaking *S. arvensis* seed dormancy (Duran & Tortosa, 1985). Similarly, application of gibberellic acid (GA_3) broke high seed dormancy in this species (Lonchamp et al., 1988; Duran & Retamal, 1989). Some wild mustard seeds germinate as soon as they mature (Mulligan & Bailey, 1975). During germination and early seedling growth, they tolerated water stress up to –0.9 MPa (Huang & Redmann, 1995). Wild mustard seeds exhibit extended seed longevity, as observed by Steiner & Ruckenbauer (1995), who reported 32% of germination after 110 years of storage at 10–15 °C with 3% moisture content.

2.5 Potential weed control applications with smoke water

Soil seed banks are the main source of seedling recruitment for weedy species (Adkins et al., 2000; Gulden & Shirtliffe, 2009). Seed dormancy is the main reason weedy species remain viable in the soil and maintain persistent soil seed banks, challenging the predictability of seedling emergence and timing for weed management and control (Adkins et al., 2000). Hence, stimulating weed seed germination can potentially provide efficient and effective weed management (Dessaint et al., 1991; Stevens et al., 2007). Current weed management practices, like crop rotation, herbicides, chaff collection, tillage, and mulching are aimed at germinable weeds (~9%) during the growing season, neglecting the more significant fraction of dormant weed seed bank (~90%) in agricultural systems (Stevens et al., 2007).

Crude smoke and smoke-derived active compounds have shown great success in stimulating weed seed germination, including herbicide-resistant weeds (Daws et al., 2007; Stevens et al., 2007; Long et al., 2010; Mojzes et al., 2015). Kępczyński (2018) reviewed smoke-responsive weed families (Asteraceae, Amaranthaceae, Boraginaceae, Brassicaceae, Caryopbyllaceae, Malvaceae, Papaveraceae, and Poaceae), focusing on the Brassicaceae family as they exhibit inherent and consistent smoke responsiveness for seed germination (Adkins & Peter, 2001; Long et al., 2010, 2011; Godakanda et al., 2020). The smoke-derived active compound karrikin (KAR1) by itself can promote light-dependent germination (Daws et al., 2007). The significant activity of smoke water or KAR1 at low concentration makes it suitable to apply in soils to control weeds (Stevens et al., 2007). It has been demonstrated that a single application of KAR1 at a low dose range of 2–20 g/ha on the

soil surface is sufficient to stimulate seed germination of *Brassica tournefortii*, *Raphanus raphanistrum*, and *Arctotheca calendula* (Stevens et al., 2007). Using smoke to stimulate germination of weedy species will ensure efficient, effective, and environmentally benign control of weed populations, minimizing chemical use (Sexsmith & Pittman, 1963; Dixon et al., 2009; Ghebrehiwot, 2010; Kamran et al., 2014). Eventually, depletion of the major dormant weed seeds in soil seed banks can be achieved in a few cropping cycles (Boutsalis & Powles, 1998).

CHAPTER 3. EFFICACY OF DIFFERENT SMOKE TYPES AND EVALUATING SMOKE WATER PRIMING ON SEED GERMINATION AMONG "FIRE-PRONE" SPECIES

3.1 Introduction

Fire is a natural disturbance in many ecosystems, and plant adaptations to fire are reflected in seed dormancy release, enhanced growth, and reproduction (Blank & James, 1998; Preston & Baldwin, 1999). Heat, ash, and smoke are fire-associated cues responsible for promoting seed germination and seedling recruitment (Henig-Sever et al., 1996; Keeley & Fotheringham, 2000). Unlike the other factors, smoke composition is highly dependent on plant material/species from which it is derived (van Staden et al., 1995b; Ren & Bai, 2016a). Plant-derived smoke solutions may have thousands of chemical compounds (Smith et al., 2003), but research has shown that smoke generated from a number of plant species can elicit similar germination responses (Baxter et al., 1995; Çatav et al., 2012). Smoke is the most striking cue that can elicit seeds of different size, shape, and life form (Dixon et al., 1995; van Staden et al., 2000).

Several species-specific pathways have been investigated for smoke-stimulated seed germination, including smoke as a chemical scarification agent by enhancing seed coat permeability (Keeley & Fotheringham, 1997; Egerton-Warburton, 1998). Smoke also interacts with plant growth regulators where smoke changes the sensitivity of seeds to phytohormones (Schwachtje & Baldwin, 2004; Nelson et al., 2009). Smoke concentration determines germination rate and seedling growth (Lloyd et al., 2000; Flematti et al., 2004; Abu et al., 2016; Ren & Bai, 2016a). Plant species from a wide range of taxa (Baxter & van Staden, 1994; Brown et al., 1994) have been shown smoke-elicited seed germination response, including those from fire-averse ecosystems (van Staden et al., 2000).

The promotive effect of smoke is influenced by various environmental factors, including temperature, heat, and light conditions (Brown et al., 1994; Ghebrehiwot et al., 2009). Seed germination and seedling growth of *Aristida junciformis* (bristle grass), *Hyparrhenia hirta* L. (thatching grass), and *Panicum maximum* (guinea grass) were consistently and positively related to temperature, suggesting that temperature interacts with smoke action on seed germination and seedling growth (Ghebrehiwot et al., 2009). Abu et al. (2016) reported that after smoke water treatments, some forage species had higher germination at 25/15 °C while others at 10/0 °C. It has also been shown that smoke solutions substitute for the light requirement of germination of Grand Rapids lettuce seeds (Drewes et al., 1995). Promotive chemical compounds in smoke solution may substitute for the light requirement of photoblastic *L. sativa* seeds in darkness (Flematti et al., 2004;

van Staden et al., 2004). In contrast, negatively photoblastic seeds can germinate in light after a smoke solution is applied (Brown, 1993a; Brown & van Staden, 1997).

Since 1990, studies have sought a suitable bioassay system to identify plants that produce smoke that promotes seed germination (Light, 2006; Flematti et al., 2008). As well, seeds of different species have been evaluated as a bioassay for smoke-stimulated seed germination. *Nicotiana attenuate* seeds were used to evaluate the efficacy of smoke extract fractions and pure chemicals that elicit seed germination (Baldwin et al., 1994). Roche et al. (1997) used *Lysinema ciliatum* as the bioassay species to test the effect of smoke solutions, while Tieu et al. (1999) used *Stylidium affine*. Drewes et al. (1995) found that the light-sensitive lettuce seeds (*L. sativa*) can quickly respond to smoke water; within 24 hours. Lettuce seeds do not germinate in darkness, a condition termed "light-sensitive," "photoblastic," or "skotodormant." However, lettuce seeds exposed to the active chemical compounds in smoke solutions overcome the light requirement during dark incubation (Drewes et al., 1995; Flematti et al., 2004; van Staden et al., 2004). The rapid germination of lettuce seeds after smoke water treatment makes this species a useful, rapid, and simple bioassay system to detect the bioactivity of different smoke solutions (Light, 2006).

Smoke solutions derived from different plant materials such as grass (Baxter et al., 1995; Clarke & French, 2005; Ghebrehiwot et al., 2009), forb and shrub species (Dayamba et al., 2008; Zuloaga-Aguilar et al., 2011), legumes (Kamran et al., 2014; Ren & Bai, 2016a), gymnosperms (Jäger et al., 1996a; Kamran et al., 2017) and even plant-derived tissue paper (Jäger et al., 1996a; Flematti et al., 2004) have been tested as seed germination stimulants. Vegetation and habitat types where plant materials originated interacts with the efficacy of smoke water solutions on seed germination. Fire-prone ecosystems such as South African fynbos (Brown, 1993b), Australian native grasslands (Dixon et al., 1995; Read & Bellairs, 1999; Clarke & French, 2005), Californian Chaparral (Keeley & Fotheringham, 1998), and fire-adapted Canadian Fescue Prairie (Romo, 2003; Gross & Romo, 2010; Ren & Bai, 2017) depend on frequent or seasonal fire events to maintain ecosystem health and function (Keeley & Fotheringham, 2000). More importantly, seed germination of fire-prone plant species was stimulated by a variety of fire-related cues, including smoke (van Staden et al., 2000). It is an ongoing debate whether smoke derived from different plants can elicit similar germination responses. van Staden et al. (1995c) found that possible stimulants varied among smoke solutions derived from *Passerina vulgaris* and *Themeda triandra*. In contrast, Grand Rapid lettuce (*L. sativa)* seeds showed a similar germination activity after being treated with smoke solutions made from *Acacia mearnsii*, *Eucalyptus grandis*, *Hypoxis colchicifolia*, *Pinus patula,* and

Themeda triandra (Jäger et al., 1996a). The fire-prone South African ecosystem differs from fire-adapted Canadian Fescue Prairie in terms of fire frequency (Anderson & Bailey, 1980), fire severity (Wright, 1971), and seasonality (Bailey & Anderson, 1978).

Smoke-elicited germination is a complex process with a convergent evolutionary characteristic (Keeley & Pausas, 2018). Phylogenetically, a vast range of plant species (*ca.* 2500) respond to smoke with enhanced germination or increased growth rate (Bradshaw et al., 2011). Asteraceae, Poaceae, Fabaceae, Restionaceae, Brassicaceae, and Rosaceae are major families with positive responses to smoke (Brown et al., 2004; Pausas & Keeley, 2009). The chemically isolated 'Karrikin' from smoke has garnered considerable interest as it promotes light-dependent germination of many agriculturally and economically important weeds (Daws et al., 2007). A substantial number of studies have shown smoke-aided weed seed bank stimulation through enhanced seed germination percentage and rate (Adkins et al., 2000; Read et al., 2000; Kępczyński et al., 2006; Daws et al., 2007; Stevens et al., 2007). Weed seeds of the family Brassicaceae are also known for their consistent smoke responsiveness with stimulated seed germination ability (Adkins & Peter, 2001; Long et al., 2010, 2011; Godakanda et al., 2020).

Studies have shown that alfalfa-derived smoke enhanced the germination of several forbs in the Fescue Prairie, but not the standard lettuce seed bioassay species (Ren, 2015). It is possible that different active compounds may be present in legumes but not in graminoids. Different bioassay species such as *Nicotiana attenuate* (Baldwin et al., 1994), *Lysinema ciliatum* (Roche et al., 1997), *Stylidium affine* (Tieu et al., 1999), *Anigozanthos manglesii* (Flematti et al., 2011a) have been used as bioactive indicators to chemically isolate compounds in smoke solutions if the conventional lettuce seed bioassay system does not respond consistently.

The objectives of this study were to: 1) compare the ability of alfalfa and wheat plant-derived smoke against South African ‘fire-ephemeral’ derived smoke types on germination responses using standard lettuce seed bioassay under different germination conditions, 2) understand how and which fire-ephemeral species respond to smoke during germination, and 3) identify and recommend potential, alternative novel bioassay species. I hypothesized that: 1) different smoke types stimulate seed germination differently, 2) temperature and light interact with smoke types to elicit germination responses differently, and 3) seed germination of fire-ephemeral species responds more positively to alfalfa and/or wheat smoke solutions.

3.2 Materials and Methods

3.2.1 Plant materials for smoke solution production

For smoke water production, alfalfa hay (*Medicago sativa* L.) and wheat straw (*Triticum aestivum* L.) were collected from Kernan Farm, Saskatoon, SK and six fire-ephemerals from fire-prone habitats in South Africa belong to graminoids in the Poaceae family (*Eragrostis curvula* (Schrad.) Nees, *Cymbopogon pospischilii* (K.Schum.) C.E.Hubb., *Melinis nerviglumis* (Franch.) Zizka), forbs in Asteraceae (*Helichrysum nudifolium* (L.) Less., *Hilliardiella capensis* (Houtt.) H. Rob., Skvarla & V. A. Funk) and in Fabaceae (*Indigofera hilaris* Eckl. & Zeyh.) were used (Table 3.1).

3.2.2 Seed sources and characteristics

For the lettuce seed bioassay test, *Lactuca sativa* L. cv. Salad Bowl achenes (hereafter referred to as seeds) were purchased from Early's Farm & Garden Centre in Saskatoon, SK. Seeds of six South African plant species were provided by Drs. Sershen Naidoo, Syd Ramdhani, at the University of KwaZulu-Natal, South Africa. Six South African species were collected between spring and summer (2016–2017; October 2016–January 2017) in KwaZulu-Natal Coastal Belt (CB3), Durban, South Africa. All these species are indigenous to South Africa and coexist in South African savannas (Mucina & Rutherford, 2006). Seed collection sites were on an undisturbed hilly grassland with a northeast facing slope (a rugby field) at Westville Campus, University of Durban Westville (26° 31′S, 28° 32′E, elevation 123 m). Mean maximum and minimum monthly temperatures for Durban (airport) are 32.6 °C and 5.8 °C January and July, respectively (Figure 3.1) (Mucina & Rutherford, 2006). Ecologically important diurnal variation in air and soil temperature of each season is summarized in Table 3.2 (Buhrmann et al*.,* 2016).

Wild mustard (*Sinapis arvensis* L.) seeds were provided by Drs. Steve Shirtliffe and Eric Johnson, Department of Plant Science, University of Saskatchewan (U of S). Seeds of *H. capensis* and *M. nerviglumis* are mostly non-dormant (ND), and *I. hilaris* seeds exhibit physical dormancy (PY). All other species studied have shallow physiological dormancy (PD) (Baskin & Baskin, 2004, 2014; Ochuodho & Modi, 2008). Seed germination and fire or smoke responsiveness for study species are summarized in Table 3.1.

Table 3.1 Germination requirements (non-dormant seeds) and fire/smoke responsiveness of seven study species.

Family (functional group)	Species	Temperature, °C (light requirements)	Fire or smoke responsiveness for seed germination	References
Asteraceae (forbs)	*Helichrysum nudifolium*	25-35(optimum); 20/10; 15/20; 20 (16 h/8 h)	Aerosol smoke responsive	Brown et al., 2003, 2004; Baskin & Baskin, 1998; Afolayan et al., 1997
	Hilliardiella capensis	30/25; 30; 25 (12 h/12 h or 14 h/10 h)	Smoke responsive	Shaukat et al., 2004; Nyamongo et al., 2009; Ghebrehiwot, 2010
Fabaceae (forbs)	*Indigofera hilaris*	20; 30/20; 31/22 (12 h/12 h or 14 h/10 h)	Fire/heat responsive	Baskin & Baskin, 2014; Sy et al., 2001; Brown et al., 2004
Poaceae (graminoid)	*Cymbopogon pospischilii*	30/20; 25; 30 (12 h/12 h or 14 h/10 h)	aerosol smoke responsive after heat	Baskin & Baskin, 2014; Read & Bellairs, 1999; Williams et al., 2014
	Eragrostis curvula	30; 35/20; 40/20 (16 h/8 h or 15 h/9 h)	Smoke responsive	Ghebrehiwot et al., 2009; Martin & Cox, 1984; Maze et al., 1993; Read & Bellairs, 1999; Ellis, 2010; Clarke & French, 2005
	Melinis nerviglumis	25; 27; 20-35 (12 h/12 h or 16 h/8 h)	Fire responsive	Kirkman et al., 2014; Stokes et al., 2011; Gorgone-Barbosa et al., 2020; Ellis, 2010
Brassicaceae (forbs)	*Sinapis arvensis*	20; 20-35 (12 h/12 h or 16 h/8 h)	Smoke responsive	Donald & Tanaka, 1993; Long et al., 2011; Stevens et al., 2007; Adkins & Peter, 2001; Goudey et al., 1987

Seeds and plant material for all species were collected at reproductive maturity (seed/fruit dehiscence) and all aerial foliage from 50 to 75 individual plants with mature seeds/fruits were harvested. Foliage was air-dried at 25 °C with seeds/fruits intact for 3–5 days, and afterwards seeds were stored in a warehouse at 15 °C until their use in 2018 or 2019. Seeds received at least one year of after-ripening after harvesting and they were cleaned by rubbing, blowing, and being passed over several metal sieves. Finally, seeds were separated using an air-column seed blower that uses an air and gravity to separate denser seeds from their lighter debris materials.

After at least one year of after-ripening at room temperature, seeds of six savanna species were hand-stripped from plants. Seeds were then cleaned and separated using metal test sieves. All cleaned seeds were kept in sealed plastic bags and stored at –20 °C until they were used for seed priming and germination experiments. Seeds were sterilized once using a 1% sodium hypochlorite (NaOCl) solution for 10 minutes and washed three times with distilled water immediately prior to the beginning of each seed priming treatment.

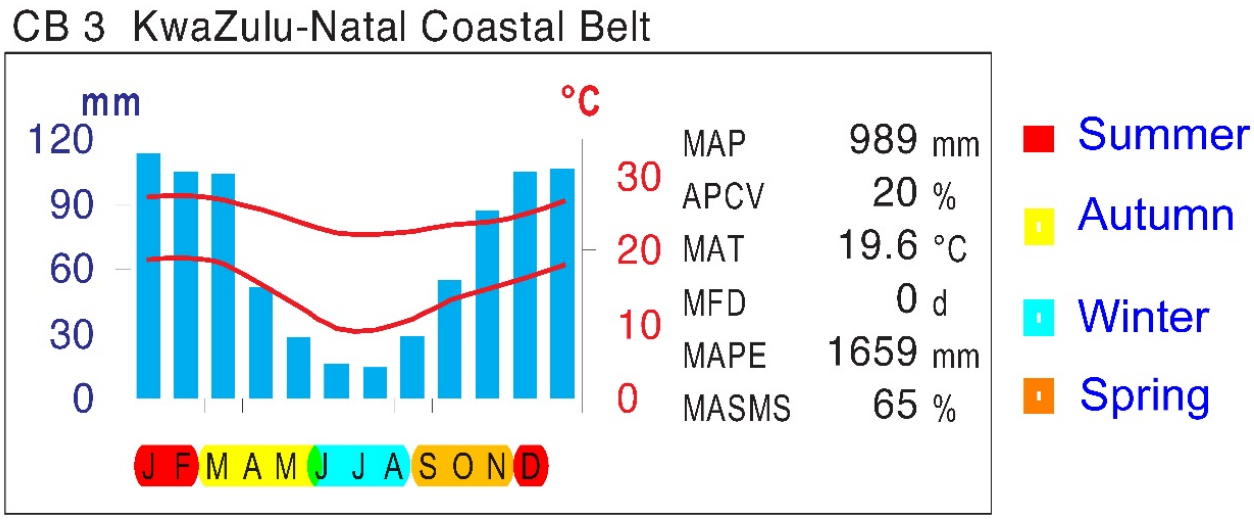


Figure 3.1 Climate diagram of KwaZulu-Natal Coastal Belt (CB3). MAP: mean annual precipitation. APCV: annual precipitation coefficient of variation. MAT: mean annual temperature. MFD: mean frost days. MAPE: mean annual potential evaporation. MASMS: mean annual soil moisture stress. Data from Mucina & Rutherford (2006).

Table 3.2 Seasonal and annual mean, absolute maximum, and absolute minimum temperatures within KwaZulu-Natal Coastal Belt (CB3).

		Annual	**Spring**	**Summer**	**Autumn**	**Winter**
Mean	Air	20.1 ± 7.6	19.2 ± 7.6	23.2 ± 7	18.2 ± 7.5	19.5 ± 7.3
	Soil	20.8 ± 4.3	19.7 ± 3.4	24.9 ± 3.3	18.2 ± 3.3	19.7 ± 3.2
Absolute maximum	Air	45.4	45.2	44.1	45	45.4
	Soil	35.9	30.7	35.9	30.5	29.9
Absolute minimum	Air	5.9	6.8	10.3	7.1	5.9
	Soil	11.5	11.8	17.2	13.2	11.5

*Adapted from Buhrmann et al. (2016).

3.2.3 Preparation of smoke solutions

A smoke-production setup constructed by Abu (2014) was used (Figure 3.2). It was comprised of an electric ring heater firmly fixed onto a wooden board. An aluminum pot containing plant subsample was kept on the ring heater, which was then covered by a 75L cylindrical metal garbage can (combustion chamber) having two opposite holes for air inflow (70–100 kPa) and smoke outflow. A heavy weight was kept on the metal garbage can to minimize gas leakage. Each smoke stock solution was produced by smoldering the relevant weight of plant subsamples one by one and continuous passing of smoke produced via the silicon tube to relevant volume of distilled water to produce each stock solution (1/1 v/v). Plant materials used were alfalfa hay, wheat straw, and six South African species (*E. curvula*, *I. hilaris*, *H. nudifolium*, *C. pospischilii*, *M. nerviglumis*, *H. capensis)*. Smoke was bubbled through distilled water for about 30 minutes (personal comm. Lei Ren & Yusuf Abu). Three replicates of stock solutions (1/1 v/v dilution) for eight plant materials were produced for each run of experiments. Each stock solution was used to prepare three serial dilutions: 1/10 v/v, 1/100 v/v, and 1/1000 v/v.

Figure 3.2 Smoke-water producing setup through smoldering of plant materials.

3.2.4 Salad Bowl lettuce bioassay

Smoke water solutions (as a seed pre-treatment tool) derived from eight different plant species (six fire-ephemerals, alfalfa, and wheat) were used to test Salad Bowl lettuce seed germination. Each smoke water stock solution (1/1 v/v) of the eight species was used to prepare three dilutions: 1/10 v/v, 1/100 v/v, and 1/1000 v/v. Five replicates of 30 seeds placed in 10 cm Petri dishes lined with two layers of Whatman #1 filter paper were used for each dilution of respective smoke water solutions in a completely randomized design (CRD). Seeds in control were imbibed in 4 mL of HPLC grade distilled water (HPLC-DW) in the dark or light (photon irradiance ~ 112 µmol photons m^{-2} s^{-1}, 400–700 nm, cool white, fluorescent tubes) at 25 °C or 25/15 °C for the duration of the experiment. In each treatment, 4 mL of smoke solution from eight species, respectively was poured onto the filter paper containing un-imbibed lettuce seeds to germinate. All germination bioassays were carried out under "green safe light" in darkness (Drewes et al., 1995). All Petri dishes were sealed in zip-lock bags and covered with two layers of aluminum foil to avoid drying out the plates and to provide darkness. The emergence of radicles was taken as the criteria for germination, which was recorded at 12-hour intervals for a total of 3–4 days, and viability of the un-germinated seeds was tested using a Tetrazolium test (ISTA, 2003). The experiment was terminated if no seed had germinated for three consecutive days. The experiment was repeated with two new replicates of stock smoke solutions. Replicates were started at 6 to 7-week intervals.

3.2.5 Fire-ephemeral seed priming and germination tests

Stock alfalfa (pH 7.43) or wheat (pH 3.42) smoke solution (1/1 v/v) produced by smoldering plants was made into three serial dilutions: 1/1000 v/v, 1/100 v/v, and 1/10 v/v. Seeds primed in HPLC-DW (0/1 v/v) were used as the control in experiments.

Both alfalfa and wheat smoke solutions were used for priming wild mustard seeds, and only alfalfa smoke solution was used to prime the six South African species. Thirty seeds (South African) or 50 seeds (wild mustard) per treatment were soaked and submerged in 10 mL of 6 priming solutions of smoke in a 50 mL centrifuge tube. Each centrifuge tube was sealed with a cap to prevent water evaporation and kept in darkness for 24 h at 20 °C. The soaked seeds were then removed and dried for one week at 25 °C on 10 cm Petri dishes lined with two layers of Whatman #1 filter paper.

The dried seeds in each Petri dishes were moistened with 4 mL of distilled water and incubated for 5–7 weeks for the South African species at 35/20 °C in 16 h light/8 h darkness or 24 h darkness and for 10 days for wild mustard seeds at 20 °C in 12 h light/dark or 24 h darkness (Conviron A1000 Plant Growth Chambers, Controlled Environments Limited). These temperatures and light regimes were selected to provide optimum conditions to maximize seed germination for the species being studied (see Table 3.1). These conditions were also similar to the summer and autumn temperatures where seed species were collected. Many tropical species have optimum germination rates above 20 °C (Teuton et al., 2004). Petri dishes were kept inside sealed transparent zip-lock bags to reduce water evaporation. Petri dishes were wrapped with two layers of aluminum foil before covering with zip-lock bags to establish 24 h darkness. Three milliliters of distilled water were added periodically to moisten the filter paper throughout the germination period.

Germination was recorded every two days or weekly, depending on species-specific seed germination speed, for up to seven weeks until no germination occurred in every treatment Petri dish for seven consecutive days. Germination of *E. curvula, M. nerviglumis,* and *S. arvensis* was counted every two days, and all other species' seed germination was counted every week for seven weeks. Seeds of *E. curvula* with both radicle (≥1 mm) and cotyledon were considered germinated. Seeds of all other South African species with a radicle ≥1 mm that emerged from seed coat were considered as germinated, whereas in *S. arvensis,* a radicle length ≥2 mm was considered as germinated. Experimental procedures and observations in 24 h darkness seed treatments were carried out necessarily under "green safe light" (540 nm, 0.3–0.5 μmol m^{-2} s^{-1}) (Drewes et al., 1995; Kulkarni et al., 2007).

Germinated seeds were counted and removed with any seed that had rotted. The final relative germination percentage was adjusted based on the viability testing (see 2.2.7) at the end of the testing period. The final relative germination ratio was calculated by dividing the total number of seeds germinated by the total number of viable seeds in a Petri plate. This ratio was adjusted to a scale of 0–100% by multiplying 100 (total germination percentage).

3.2.6 Seed Viability Testing

The viability of ungerminated lettuce seeds was evaluated using Tetrazolium Chloride (TZ) tests. Seeds were submerged in distilled water for 6 hours at 20 °C. Their embryos were then exposed by gently pressing seed coat (lettuce) at one side of the outer cotyledon. TZ solution (1.0%) was used to stain the cotyledon for 6 h at 30 °C. Seed viability was determined by observing the coloration of the cotyledon and radicle (ISTA, 2003).

At the end of primed seed germination test, the viability of small un-germinated South African seeds was tested by checking the rigidity of seeds through the "squeezing/punching" method or by opening the seed coat with a needle and examining the embryo under a dissecting microscope. Seeds were regarded as viable if they were still firm in texture as determined by punching/squeezing the non-germinated seeds. If needle-opened seeds had white and firm embryos, they were considered viable. Soft and brownish-yellow embryos were considered non-viable (Fan et al., 2016).

The viability of un-germinated wild mustard seeds was evaluated using Tetrazolium Chloride (TZ) tests. Seeds were submerged in distilled water for 18 hours in water at 20 °C. Then their embryos were exposed by incising seed coat crosswise at one side of the outer cotyledon. TZ solution (1.0%) was used to stain the cotyledon for 18 h at 30 °C. Seed viability was determined by observing the coloration of the cotyledon and radicle (ISTA, 2003). Seeds were regarded as viable if they stained a maximum area.

3.2.7 Estimating germination rates (T_{50}, T_{25}, or T_{10}) using *Chapman-Richard's* growth function

The Chapman-Richard's growth function has been used extensively to describe sigmoidal growth forms such as cumulative germination time courses (Richards, 1959; Qiu et al., 2006) with the following equation:

$$g = b_1 (1–\exp (–b_2 t))^{b_3} \quad \text{(Eq. 3.1)}$$

where g = germination percentage, t = time, b_1 = the asymptote, b_2 = rate parameter and b_3 = shape parameter (Qiu et al., 2006; Abu et al., 2016). b_1, b_2 and b_3 are constants. A given treated seed population has a population-specific sigmoidal growth curve with a specific asymptote (constant b_1) for its defined maximum germination percentage. The other two constants (b_2 and b_3) define the shape of the curve.

Nonlinear regression was performed for the Chapman-Richard's growth function in the R software language using 'nlsLM' function within the package 'minipack.lm version 1.2-1'(R Development Team, 2020: R v. 3.6.3). Constants (b_1, b_2 and b_3) were estimated by solving for the nonlinear least squares via a modification of the Levenberg-Marquardt algorithm (https://cran.r-project.org/, 2016). Time to 50% germination of whole sub-populations was estimated and extracted (hereafter referred to as the germination rate) through Chapman-Richard's nonlinear curve fitting in R software. In consideration of model complexity and accuracy, the best model was selected among candidate models with possible b_1, b_2 and b_3 constants using the second-order AIC (AICc), which is a refined version of AIC for a small sample size (Burnham et al., 2011; Symonds & Moussalli, 2011). AICc was calculated using the 'AICcmodavg' (version 2.3-1) package in R.

$$\text{AICc} = 2k + n\left[\ln\left(\frac{RSS}{n}\right)\right] + \frac{2k^2+2k}{n-k-1} \qquad \text{(Eq. 3.2)}$$

where *k* is the number of fitted parameters in the model, n is the sample size of each sub-populations, and RSS is the residual sum of squares in the model.

Even though calculating an R^2 is not appropriate for nonlinear regression, the "goodness of fit" of the model was determined using the Pseudo-R^2 value in R software as follows.

$$\text{Pseudo-R2} = 1 - \text{SS(Residual)}/\text{SS}(\text{Total}_{\text{Corrected}}) \qquad \text{(Eq. 3.3)}$$

where SS (Residual) = deviance or (observed value – predicted value); SS (Total) = (observed value – mean). SS stands for sum of squares.

3.2.8 Data analysis

All statistical analyses were performed using R v. 3.6.3 (R Development Team, 2020). All experiments followed a randomized-complete-block design (RCBD). Final germination percentage and germination rate (T_{10} or T_{25} or T_{50}) were analyzed using General Linear Mixed Model (GLM) procedure within package 'lme4' version 1.1-23. Main effects and possible interactions of temperature, light regimes, smoke types, and dilutions were used as independent variables to evaluate their effects on seed germination percentage and germination rate. Replicates and runs were factored into the model as random effects. Final germination data were analyzed using Analysis of Variance (ANOVA) in the mixed model. Data normality was tested with R software. Germination data were arcsine square root transformed before ANOVA. Treatment means were separated using function 'difflsmeans,' considering the significance level of α=0.05. All analysis tables are available in Appendices A1 – A3.

3.3 Results

3.3.1 Effect of smoke types on Salad Bowl lettuce seed germination

Seed germination of Salad Bowl lettuce was affected by the interaction of smoke type × smoke dilution in both darkness ($P < 0.001$) and 12 h light - 12 h dark ($P < 0.001$) germination conditions at a constant 25 °C (Figure 3.3) and alternating 25/15 °C (Figure 3.4), respectively. Salad Bowl lettuce seeds treated with HPLC-DW (control) at 25 °C showed stable germination around 53% in 24 h dark condition and around 84% in 12 h light / 12 h darkness at constant 25 °C (Figure 3.3). However, at alternating 25/15 °C temperature, seeds treated with HPLC-DW (control) in darkness showed consistently lower germination (~36%) and in light condition showed more or less similar germination (~90%) in comparison with germination at 25/15 °C (Figure 3.4).

Seeds treated with concentrated 1/1 and 1/10 v/v smoke dilutions prepared from all types of plant materials or plant functional groups reduced lettuce seed germination significantly ($P<0.05$) compared to the distilled water control in both 24 h darkness and 12 h light/12 h darkness at a constant 25 °C (Figure 3.3). However, there was one exception. At alternating 25/15 °C, lettuce seed germination was increased significantly after treatment with 1/10 v/v dilution of alfalfa and three graminoids (wheat, *Eragrostis, Melinis*) smoke types (Figure 3.4).

At least diluted smoke solutions stimulated lettuce seed germination only at darkness but varied between seed incubation temperatures and based on which plant material was used to produce smoke solutions. For example, diluted 1/100 and 1/1000 v/v smoke solutions derived from all plant species increased seed germination significantly in 25/15 °C darkness among all other germination conditions (Figure 3.4 a). Also, the most dilute 1/1000 v/v smoke solutions made from wheat straw and *E. curvula* significantly increased lettuce seed germination in 25 °C darkness by 14% and 18%, respectively. (Figure 3.3 a).

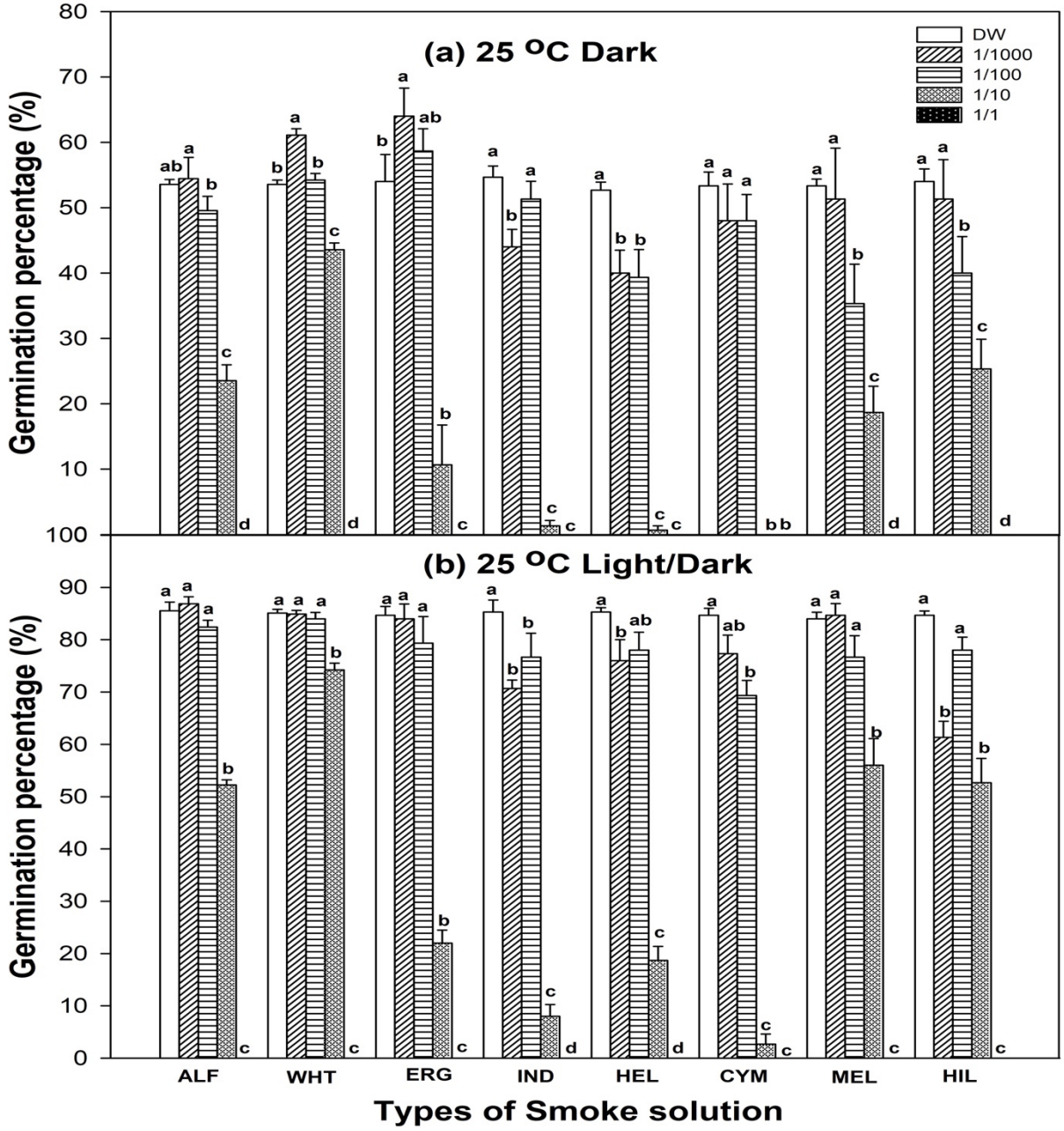


Figure 3.3 Germination percentage of Salad Bowl lettuce (*L. sativa*) seeds after treatment with HPLC-DW (HPLC grade distilled water) or serial dilutions of 8 aqueous smoke solutions (ALF- alfalfa, WHT-wheat, ERG- *E. curvula*, IND- *I. hilaris*, HEL- *H. nudifolium*, CYM- *C. pospischilii*, MEL- *M. nerviglumis*, HIL- *H. capensis*) at 25 °C in (a) 24 h darkness and (b) 12 h light–darkness cycle for 1 day. Means with different letters indicate significant differences (P≤0.05) within each smoke type (means ± S.E., number of replicates; n = 15).

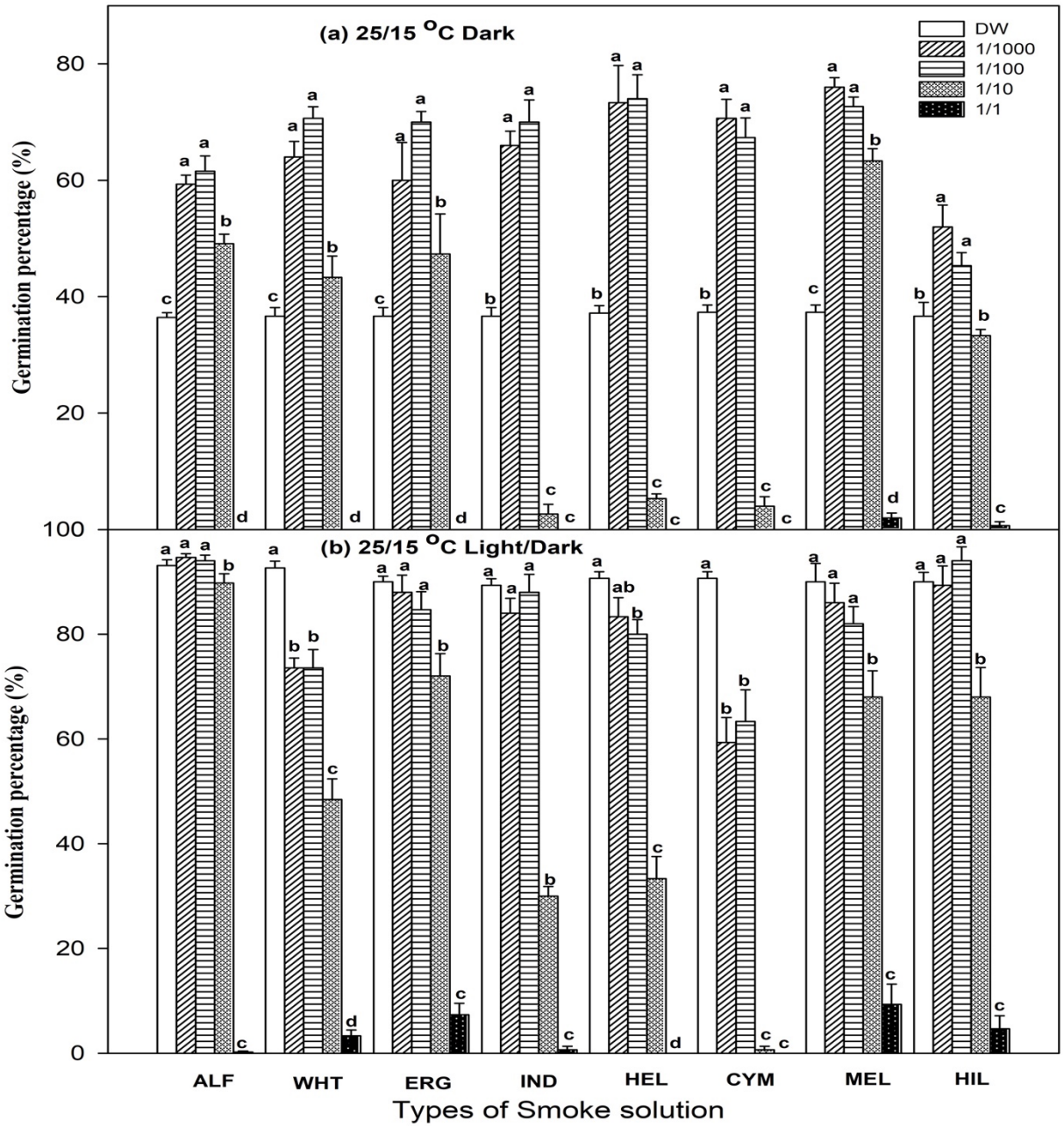


Figure 3.4 Germination percentage of Salad Bowl lettuce (*L. sativa*) seeds after treatment with HPLC-DW (HPLC grade distilled water) or serial dilutions from eight aqueous smoke solutions (ALF-alfalfa, WHT-wheat, ERG- *E. curvula*, IND- *I. hilaris*, HEL- *H. nudifolium*, CYM- *C. pospischilii*, MEL- *M. nerviglumis*, HIL- *H. capensis*) at alternating 25/15 °C in (a) 24 h darkness and **(**b) 12 h light–darkness cycle for 1 day. Means with different letters indicate significant differences (P≤0.05) within each smoke type. (means ± S.E., number of replicates; n = 15).

3.3.2 Effect of smoke solution concentration (dilutions), incubation temperature, light regimes, and their interactions on seed germination of Salad Bowl lettuce seeds

Analysis of variance showed that germination percentage of this bioassay species was significantly ($P < 0.001$) different between smoke dilutions, light regimes, temperatures, and most of their interactions (Appendix Table A.1.1.). However, the differences attributable to temperature and light regimes were not significant ($P \leq 0.05$), indicating that the germination response of seeds at both 25 °C and 25/15 °C temperatures was consistent under both light conditions. Smoke generated from different plant functional groups (i.e., South African native forbs, graminoids) promoted lettuce seed germination differently. In full darkness, smoke generated from only two graminoids (i.e., wheat and *Eragrostis*) at 25 °C (Figure 3.3 a) and all type of functional groups at 25/15 °C (Figure 3.4 a) significantly enhanced lettuce seed germination.

The interaction between smoke produced from different plants and their serial dilutions was highly significant ($P \leq 0.001$), suggesting that the effect of smoke dilution was not consistent among smoke types of different plant species (Appendix Table A.1.1.). The diluted 1/1000 v/v and 1/100 v/v smoke solutions from leguminous and grass species increased the seed germination percentage compared to that of concentrated 1/10 v/v and 1/1 v/v smoke solutions (Appendix Table A.1.1.). At 1/1000 v/v dilution, alfalfa, *Eragrostis,* and *Melinis* smoke types enhanced seed germination compared to other smoke types. Interestingly, even at a higher smoke concentration in 1/10 v/v, alfalfa and wheat smoke were effective in enhancing seed germination percentage (>50%) compared to that of other South African smoke types. The exception was *Melinis* smoke.

Being exposed to light during incubation increased seed germination significantly ($P \leq 0.001$) compared to total darkness regardless of the smoke type (Figure 3.3 and 3.4). The interaction between light regimes and smoke types was significant ($P \leq 0.001$), indicating that the effect size of light was not consistent among smoke types (Appendix Table A.1.1.). For example, after treating seeds with smoke from *Cymbopogon*, seeds showed a smaller light effect than that of the alfalfa smoke treated seeds on germination. Surprisingly, similar to most South African smoke types, the alfalfa smoke solution was more effective at alternate 25/15 °C than at constant 25 °C in promoting seed germination (Figure 3.3 and 3.4), indicating that different smoke types interact with different temperature regimes to stimulate seed germination.

3.3.3 Effect of alfalfa and wheat smoke solutions on seed germination rate of Salad Bowl lettuce seeds

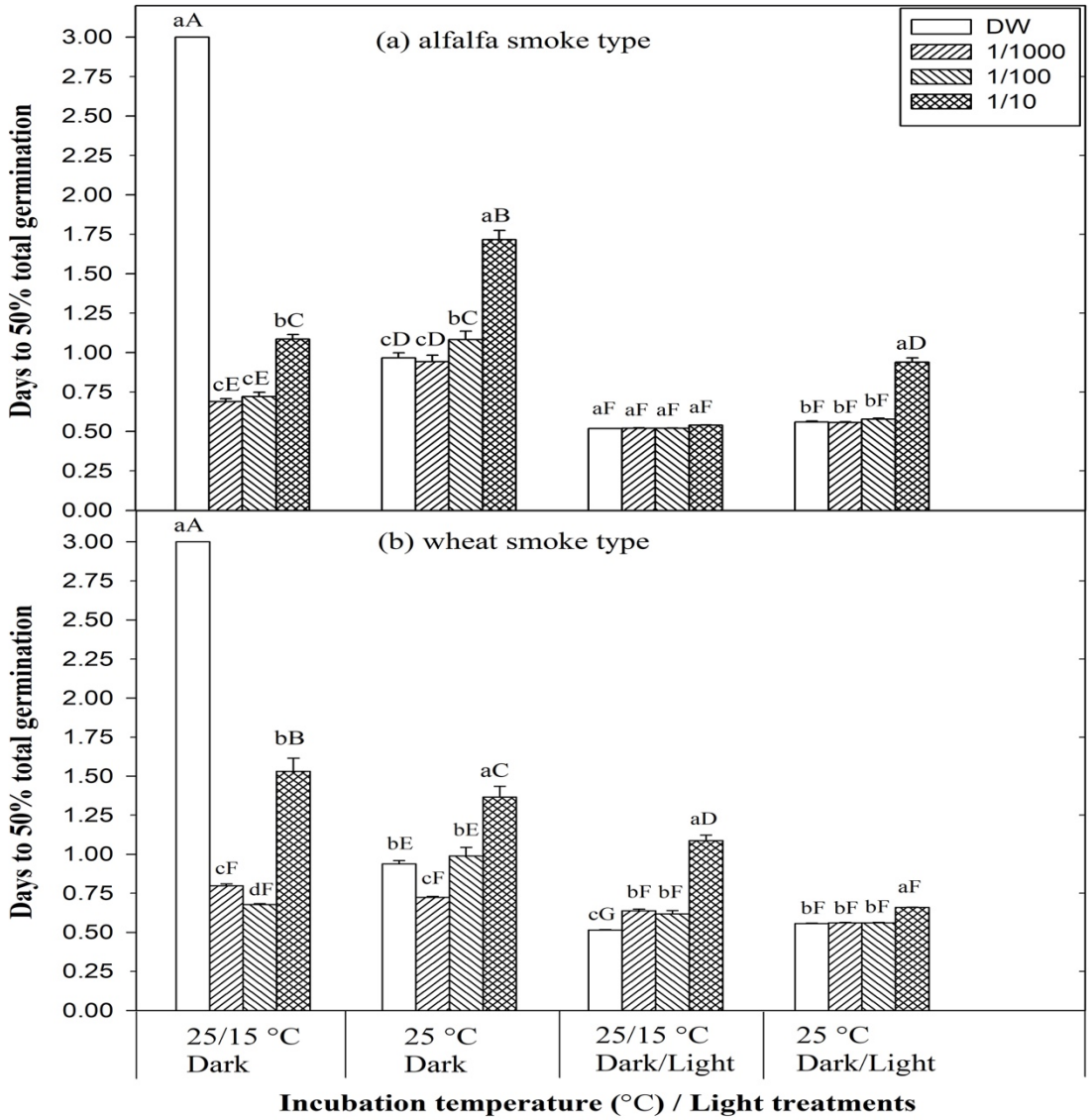


Figure 3.5 Days to 50% of total germination of Salad Bowl lettuce (*L. sativa*) seeds after treatment with (a) alfalfa or (b) wheat smoke dilutions and incubation at alternating 25/15 °C or constant 25 °C conditions, respectively. DW (HPLC grade distilled water) or serial dilution of each smoke solution by volume/volume (v/v) (1/1000, 1/100, 1/10). Means with different lower-case letters (within temperature-light regimes) or different upper-case letters (between same temperature or same light regimes) had significantly different ($P \leq 0.05$) germination rates (means ± S.E., n = 5 or 10).

Seed germination rate determined by T_{50} was not significantly different between alfalfa and wheat smoke types ($P > 0.05$), indicating that both alfalfa and wheat smoke types had a similar effect on germination rates (Appendix Table A.1.4.). Salad Bowl lettuce seeds that were treated with HPLC-DW control at 25/15 °C darkness, showed significantly slower germination compared to that of all other germination conditions (Figure 3.5). Specifically, fast seed germination was only observed in darkness, not in light, as a result of smoke solution treatments.

The three-way interaction of smoke type, incubation temperature, and smoke dilutions were significant ($P < 0.001$), indicating that the germination rate did not follow a consistent pattern in response to smoke type and their dilution across incubation temperatures (Appendix Table A.1.4.). Because of this three-way interaction effect and the distinct ecological significance of smoke source including from a leguminous forb and a graminoid wheat, I compared mean germination rates within each smoke type separately (*a* and *b* in Figure 3.5). As a general pattern, seeds germinated at a slower speed (> 3 days to reach 50% germination) after treatment in 1/1 v/v dilution of alfalfa or wheat smoke solution at all temperatures and light regimes (data not shown in Figure 3.5). Interestingly, only at 25/15 °C darkness, concentrated 1/10 v/v dilution of either wheat or alfalfa treated seeds germinated much faster ($P < 0.05$) compared to the distilled water control.

At 25/15 °C darkness, a 1/100 v/v dilution of either wheat or alfalfa treated seeds germinated faster than that at 25 °C. However, at 25 °C darkness, seeds germinated faster after treatment with the most dilute 1/1000 v/v smoke solution from wheat only (Figure 3.5 b). At 25/15 °C darkness, 1/1000 v/v smoke solutions either from alfalfa or wheat increase the seed germination speed (Appendix Table A.1.3–1.4).

3.3.4 Effect of smoke water priming on seed germination and germination rate of South African fire-ephemerals

Six South African fire-ephemeral seeds responded significantly ($P \leq 0.05$) to alfalfa smoke water priming treatments in terms of total germination percentage and germination rate (T_{10} or T_{25} or T_{50}) in a species-specific pattern (Appendix Table A.2.1–2.6). Among the South African species, *E. curvula* and *H. capensis* maximized germination (Figure 3.6 a). Relative to the distilled water control, germination was faster in all South African seeds primed in alfalfa smoke in both germination rate estimates except with *H. nudifolium* (Table 3.3).

3.3.4.1 Eragrostis curvula

Total seed germination of *E. curvula* responded significantly to alfalfa smoke priming treatments in 24-hour darkness ($P < 0.001$) and 16/8 h alternating light/ dark ($P < 0.001$), respectively at 35/20 °C. There was no significant effect of light regimes or light × priming treatment ($P > 0.05$) on *E. curvula* seed germination, indicating that the smoke-mediated seed germination of this species was independent of light condition (Figure 3.6 a).

The total germination percentage of *E. curvula* seeds that were primed in distilled water (control) was around 72% in both light regimes. Seeds primed in 1/100 v/v dilution increased their germination by 15% and 18% compared to the control in 24 h darkness and 12 h light/dark light, respectively. However, seeds primed in a 1/1 v/v smoke dilution reduced the germination by 47% and 43% relative to the control in 24 h darkness or 12 h light/dark light, respectively. The high concentration of alfalfa smoke solution has negative effects on germination in tested conditions compared with the control.

Germination rate was affected by the interactive effect of priming dilutions and light regimes ($P<0.001$) at 35/20 °C. Germination rate estimates of T_{10} and T_{25} did not show a significant increase or decrease in seeds primed with smoke solutions compared to the controls in both light regimes (Table 3.3). However, with the exception of. *E. curvula* seeds primed in 1/1 v/v dilution showed a significantly ($P<0.05$) slow germination in both light regimes (Table 3.3). Among all species, only *E. curvula* seeds primed in smoke dilutions, except the 1/1 v/v dilution achieved the 50% germination. The T_{50} was significantly ($P<0.05$) lower in seeds primed with 1/100 v/v dilution under both light regimes, indicating that the smoke-stimulated faster seed germination was insensitive to seed incubating light condition.

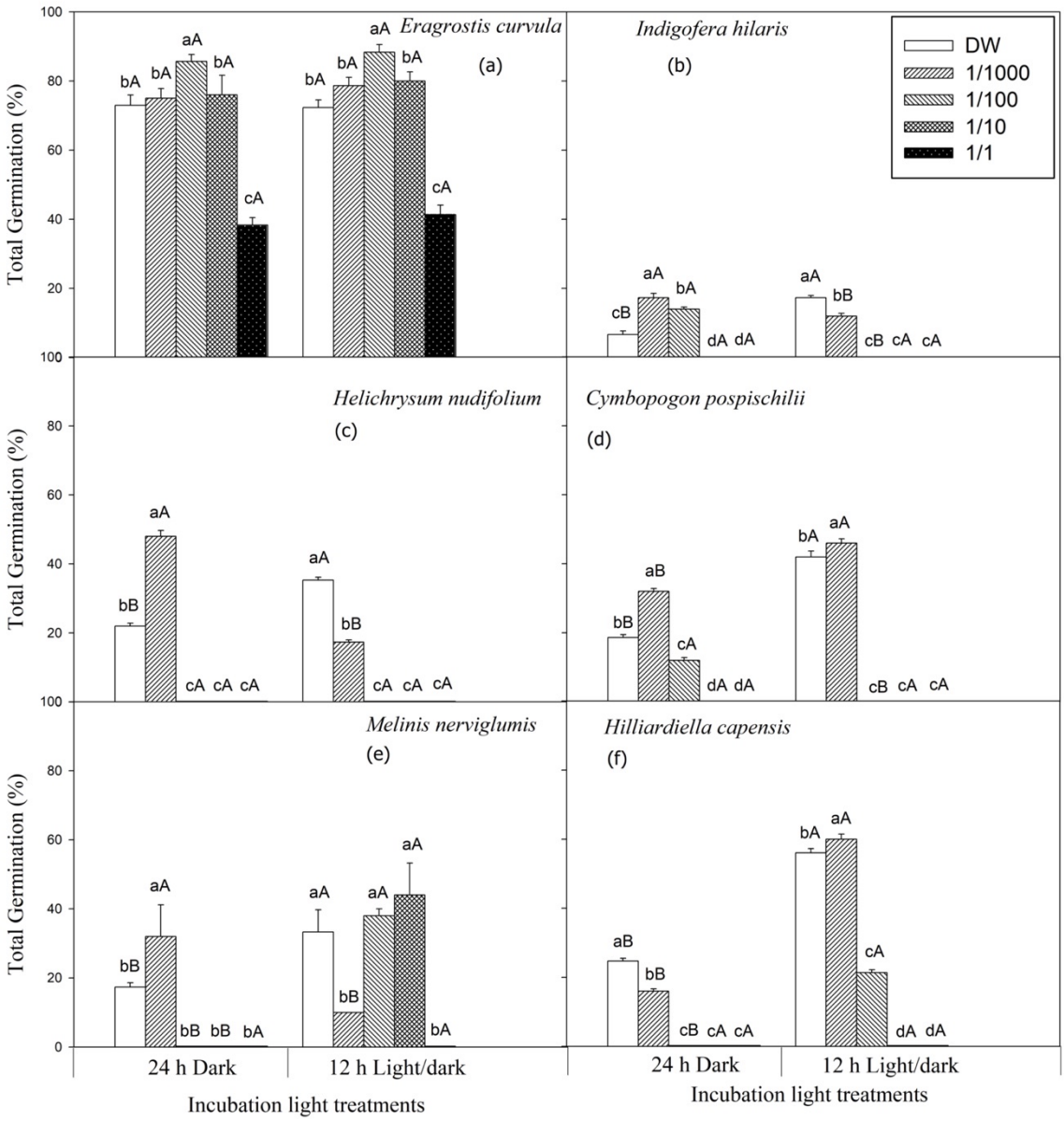


Figure 3.6 Total germination (%) of six South African fire-ephemeral seeds primed in serial dilutions of alfalfa aqueous smoke water and incubated at alternate 35/20 °C in 24 h darkness or 16h/8 h light/dark alteration for seven weeks. (DW: HPLC grade distilled water as the control). Means with different lower-case letters (within a light regime) or different upper-case letters (between light regimes with same dilutions) indicate total germination of primed seeds were significantly different ($P \leq 0.05$) (means ± S.E., n = 5 or 10).

3.3.4.2 Indigofera hilaris

Total germination percentage was affected by the interaction of priming dilution and light regime ($P<0.001$). *I. hilaris* germinated the least among species in both controls and smoke primed seeds; albeit seeds still showed a greater ($P<0.05$) germination (by 62% and 52%) when primed in 1/1000 v/v and 1/100 v/v, respectively, compared to the control under 24 h darkness (Figure 3.6 b). However, germination of seeds primed in 1/1000 v/v was reduced by 31% compared to the control under a diurnal light regime. Germination of seeds primed in 1/1000 v/v was significantly higher in darkness compared to under diurnal light, indicating that the smoke responsiveness of this species was more likely during darkness. In addition, seeds primed in 1/1 v/v and 1/10 v/v smoke dilutions reduced germination to 0% relative to controls in both light regimes, suggesting that higher smoke concentrations have toxic effects on germination in *I. hilaris*.

Germination rate was regulated by the interactive effect of priming dilution and light regime ($P<0.001$). In any of the smoke solution priming conditions, seeds did not achieve more than 18% germination, which enabled only T_{10} to be calculated as the rate parameter. At 24 h darkness, germination was faster (lower T_{10}) in seeds primed in 1/1000 and 1/100 v/v smoke solutions compared to the control. At 12 h light/dark light, a faster seed germination was observed for seeds primed in 1/1000 v/v compared to the control (Table 3.3). However, there was no significant rate difference between light regimes in seeds primed in 1/1000 v/v ($P>0.05$).

3.3.4.3 Helichrysum nudifolium

Total germination percentage was affected by the interaction of priming dilution and light regime ($P<0.001$). Only the seeds primed in 1/1000 v/v dilution showed an increase ($P<0.05$) in germination by 54% compared to the control under 24 h darkness (Figure 3.6 c). In contrast, under diurnal light, seeds primed in 1/1000 v/v showed a reduction in germination of 51% compared to the control. Germination of seeds primed in 1/1000 v/v was significantly higher in darkness compared to diurnal light, indicating that the smoke responsiveness of this species was more likely during darkness. In addition, seeds primed in 1/1, 1/10 and 1/100 v/v smoke dilutions reduced germination to 0% relative to the controls in both light regimes, showing that the higher smoke concentrations have a negative effect on seed germination of *H. nudifolium.*

Germination rate (only in T_{25}) was controlled by the interactive effect of priming dilutions and light regime ($P<0.001$) (Table 3.3). However, germination of *H. nudifolium* seeds primed in

smoke solution was not faster (in either T_{10} or T_{25}) compared to the controls in both light regimes (Table 3.3). In 12 h light/dark, germination was much slower (higher T_{25}) in primed seeds compared to the control in 24 h darkness.

Table 3.3 Germination rate, T_{10} or T_{25} or T_{50} (time to 10% or 25% or 50% of total germination in weeks) of South African fire-ephemeral seeds primed in alfalfa smoke water (mean ± S.E., n = 5 or 10).

Species	T_{10} or T_{25} or T_{50} (weeks) with smoke concentration effects					
		DW	1/1000 v/v	1/100 v/v	1/10 v/v	1/1 v/v
Eragrostis curvula	T_{10} -D	1.28 ± 0.0 ^{bA}	1.28 ± 0.0 ^{bA}	1.28 ± 0.0 ^{bA}	1.28 ± 0.0 ^{bA}	2.30 ± 0.0 ^{aA}
	T_{10} -L/D	1.28 ± 0.0 ^{bA}	1.36 ± 0.0 ^{bA}	1.24 ± 0.0 ^{bA}	1.30 ± 0.0 ^{bA}	1.98 ± 0.0 ^{aA}
	T_{25} -D	1.50 ± 0.0 ^{bA}	1.50 ± 0.0 ^{bA}	1.46 ± 0.0 ^{bA}	1.50 ± 0.0 ^{bA}	3.24 ± 0.1 ^{aA}
	T_{25} -L/D	1.63 ± 0.0 ^{bA}	1.63 ± 0.0 ^{bA}	1.56 ± 0.0 ^{bA}	1.63 ± 0.0 ^{bA}	2.74 ± 0.1 ^{aB}
	T_{50} -D	1.88 ± 0.3 ^{aB}	1.86 ± 0.0 ^{aA}	1.74 ± 0.0 ^{bA}	1.90 ± 0.0 ^{aA}	-
	T_{50} -L/D	2.28 ± 0.0 ^{aA}	2.12 ± 0.0 ^{aA}	2.04 ± 0.0 ^{bA}	2.16 ± 0.0 ^{aA}	-
Melinis nerviglumis	T_{10} -D	1.76 ± 0.3 ^{aB}	1.52 ± 0.0 ^{aB}	-	-	-
	T_{10} -L/D	2.26 ± 0.0 ^{bA}	3.45 ± 0.0 ^{aA}	1.08± 0.0 ^{c}	0.80 ± 0.0 ^{c}	-
	T_{25} -D	-	3.38 ± 0.1 ^{a}	-	-	-
	T_{25} -L/D	2.72 ± 0.1 ^{a}	-	2.48 ± 0.1 ^{a}	1.36 ± 0.1 ^{b}	-
Indigofera hilaris	T_{10} -D	3.56 ± 0.3 ^{aA}	1.78 ± 0.0 ^{bA}	2.06 ± 0.0 ^{b}	-	-
	T_{10} -L/D	2.52 ± 0.0 ^{aB}	1.68 ± 0.0 ^{bA}	-	-	-
Cymbopogon pospischilii	T_{10} -D	4.48 ± 0.0 ^{aA}	1.30 ± 0.0 ^{cA}	3.72 ± 0.0 ^{b}	-	-
	T_{10} -L/D	1.20 ± 0.0 ^{aB}	0.80 ± 0.0 ^{bB}	-	-	-
	T_{25} -D	-	4.75 ± 0.0 ^{aA}	-	-	-
	T_{25} -L/D	1.82 ± 0.0 ^{a}	1.36 ± 0.0 ^{bB}	-	-	-
	T_{10} -D	1.67 ± 0.0 ^{bA}	3.06 ± 0.0 ^{aA}	-	-	-

Hilliardiella capensis	T_{10} -L/D	0.95 ± 0.0 ^{bB}	0.85 ± 0.0 ^{bB}	1.30 ± 0.0 ^{a}	-	-
	T_{25} -D	6.40 ± 0.0 ^{aA}	-	-	-	-
	T_{25} -L/D	1.66 ± 0.0 ^{aB}	1.24 ± 0.0 ^{b}	-	-	-
Helichrysum nudifolium	T_{10} -D	0.86 ± 0.0 ^{bA}	1.34 ± 0.0 ^{aA}	-	-	-
	T_{10} -L/D	1.05 ± 0.0 ^{bA}	1.56 ± 0.0 ^{aA}	-	-	-
	T_{25} -D	1.82 ± 0.0 ^{bB}	2.32 ± 0.0 ^{a}	-	-	-
	T_{25} -L/D	2.86 ± 0.0 ^{aA}	-	-	-	-

D; dark incubation, L/D; 16 h L / 8 h D alternation "-" mark represents seeds primed in smoke that did not achieve 10% or 25% or 50% germination.
a, b, c = Means within a row with the same lower-case letter are not significantly different (P>0.05).
A, B = For a given germination rate parameter (T_{10} or T_{25} or T_{50}), means within a column (between D & L/D) with the same upper-case letter are not significantly different (P>0.05).

3.3.4.4 Cymbopogon pospischilii

Total germination percentage was affected by the interaction of priming dilution and light regime (P<0.001). Seeds primed in 1/1000 v/v showed an increase (P<0.05) in germination by 42% in 24 h darkness and by 9% in the diurnal light regime (Figure 3.6 d). The germination of seeds primed in 1/1000 v/v, was significantly higher under the diurnal light than that of in 24 h darkness. In contrast, in both light regimes, seeds primed in 1/1, 1/10 & 1/100 v/v dilutions, showed a significant reduction in germination, indicating the toxic effects of these smoke solutions.

Germination rate responses were explained by the interactive effect of priming dilution and light regime (P<0.001). In 24 h darkness, germination was faster (lower T_{10}) in seeds primed in 1/1000 v/v and 1/100 v/v compared to the control. However, the germination of seeds primed in 1/1000 v/v was much more rapid (in both T_{10} & T_{25}) under diurnal light condition than in 24 h darkness (Table 3.3).

3.3.4.5 Melinis nerviglumis

Total germination percentage was affected by the interaction of priming smoke dilution and light regime ($P<0.001$) (Figure 3.6 e). In 24 h darkness, seeds primed in the 1/1000 v/v dilution increased germination significantly (by 46%) compared to the control in darkness. However, seeds primed in the same 1/1000 v/v dilution, reduced the total germination significantly (by 70%) compared to the control in diurnal light ($P<0.05$). As well, the germination of seeds primed in 1/1000 v/v was significantly higher in darkness than in the diurnal light regime (Figure 3.6 e). Seeds primed in 1/1, 1/10 and 1/100 v/v smoke dilutions reduced the germination to 0% relative to the control in 24 h darkness, suggesting that there were toxic effects at higher smoke concentrations that negatively affect seed germination in darkness. However, during under diurnal light conditions, this toxic effect of higher smoke concentrations has been overcome.

Germination rate was affected by the interaction of priming dilution and light regime ($P<0.001$). In 24 h darkness, germination was faster (lower T_{10}) only in 1/1000 v/v primed seeds compared to the control. However, in diurnal light, germination was more rapid (lower T_{10}) in seeds primed in both 1/100 and 1/10 v/v compared to the control (Table 3.3). Seeds primed in the 1/1000 v/v dilution, germinated much more quickly (lower T_{10}) in darkness than that of under diurnal light ($P<0.05$).

3.3.4.6 Hilliardiella capensis

Total germination percentage was affected by the interaction of priming dilution and light regime ($P<0.001$). In 24 h darkness, seeds primed in the 1/1000 v/v dilution showed a significant reduction in germination ($P<0.05$) compared to the control in darkness (Figure 3.6 f). Seeds primed in the same 1/1000 v/v dilution showed a significant increase in germination compared to the control under diurnal light, indicating that light during incubation has a variable effect on smoke-mediated seed germination. Germination of seeds primed in 1/1000 v/v was significantly higher under diurnal light ($P<0.05$) than in 24 h darkness. In addition, seeds primed in 1/1 v/v and 1/10 v/v smoke dilutions reduced the germination to 0% (Figure 3.6 f) compared to the controls in both light regimes.

Germination rate was controlled by the interactive effect of priming dilution and light regime ($P<0.001$). At 24 h darkness, germination was much slower (higher T_{10}) in seeds primed in 1/1000 v/v dilution compared to the control. However, germination was more rapid under diurnal light

regimes after seeds were primed in the same 1/1000 v/v dilution compared to the control (Table 3.3). Seeds primed in 1/1000 v/v, germinated much more quickly (lower T_{10}) under diurnal light than in darkness ($P<0.05$). Under diurnal light, seeds only primed in the 1/1000 v/v dilution achieved 25% of total germination with faster germination (lower T_{25}) compared to the control.

3.3.5 Effect of smoke water priming on Wild mustard seed germination and germination rate

Seed germination in *S. arvensis* responded significantly to smoke priming treatment ($P<0.001$), smoke type ($P<0.001$), and the interactions of priming × light ($P<0.01$) and priming × smoke type ($P<0.001$) (Appendix Table A.2.7).

Unlike South African fire-ephemerals, wild mustard seeds exhibit a significant increase in total germination percentage, even after being primed with high concentration dilutions of either alfalfa or wheat smoke compared to the control (Figure 3.7). In 24 h darkness, seeds primed in a 1/1 v/v dilution showed a significant increase in germination ($P<0.05$) compared to that under diurnal light, indicating that different incubation light conditions interact with smoke to stimulate seed germination of this weedy species under specific smoke dilution (Figure 3.7).

Germination was affected by the priming dilution and smoke type ($P<0.001$). In general, germination was positively related to smoke solution concentration. Seeds primed in all dilutions made from either alfalfa hay or wheat straw increased wild mustard seed germination except the 1/1000 v/v dilution produced from alfalfa and wheat straw in light (Figure 3.7). Germination was significantly ($P<0.05$) increased in seeds primed in the 1/1 v/v dilution of alfalfa smoke solution in darkness (11%) over that in diurnal light. In all other conditions, germination was similar between light and dark regimes, for a given smoke type.

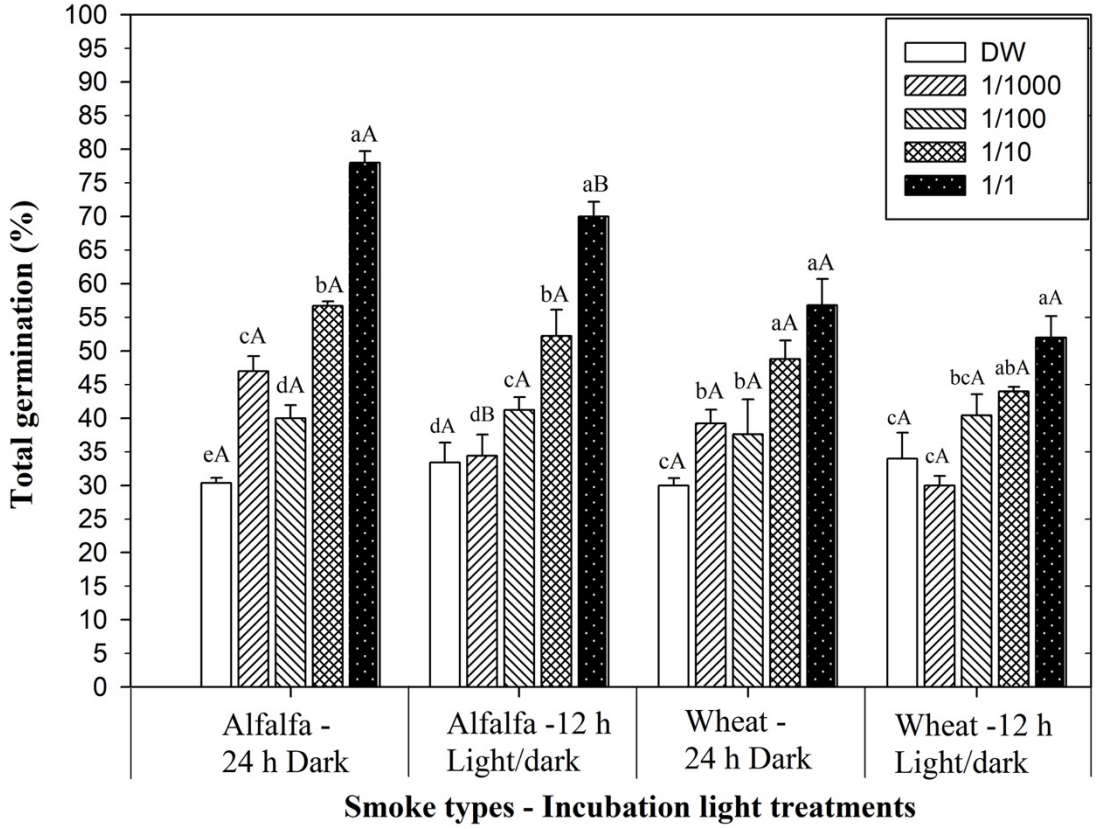


Figure 3.7 Total germination percentage of wild mustard (*S. arvensis*) seeds after priming with distilled water or different dilutions of alfalfa or wheat aqueous smoke solutions and incubation at 20 °C in 24 h darkness or 12 h light/dark alternation. (DW: HPLC grade distilled water as the control). Means with different lower-case letters (within a smoke type-light regime) or different upper-case letters (between light regimes of the same smoke type) indicate total germination of primed seeds were significantly different ($P \leq 0.05$) (means ± S.E., n = 15).

On day 1, wild mustard seeds showed a significant dark germination increment ($P<0.001$) only among seeds primed in a 1/10 v/v dilution of alfalfa smoke solution (by 71%) relative to the distilled water control (Figure 3.8). However, on day 1, no germination was observed for seeds primed in 1/1 v/v dilution. After three days, seeds primed in 1/1 v/v dilution showed the highest ($P<0.001$) germination; a 120% increase (Figure 3.11 A). Except for the 1/100 v/v dilution, seeds primed in all other smoke water dilutions maintained a significant seed germination capacity ($P<0.001$) and the pattern across day 5, day 7, and day 10 (Figure 3.8).

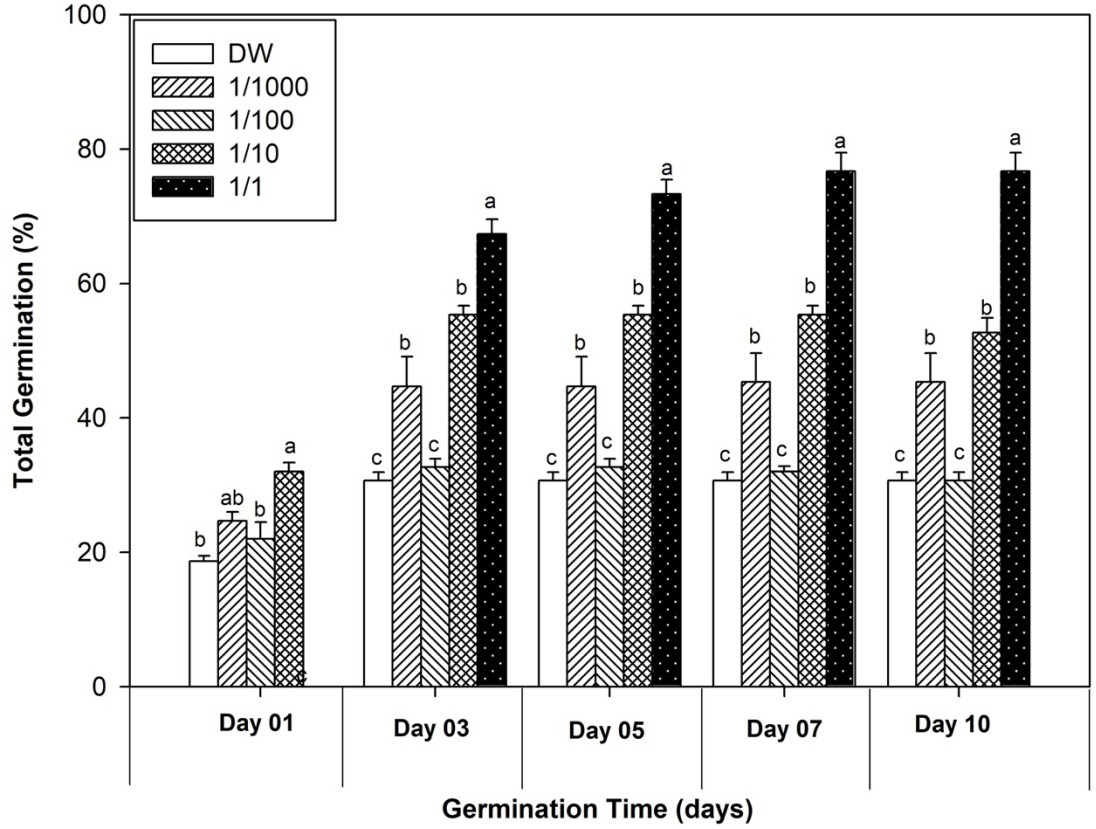


Figure 3.8 Total germination of wild mustard (*S. arvensis*) seeds after priming with distilled water or different dilutions of alfalfa aqueous smoke solutions, incubated at 20 °C in 24 h darkness for 10 days. Means with different letters indicate total germination of treated seeds was significantly different (P≤0.05) within each day. (DW: HPLC grade distilled water as the control. (means ± S.E., n = 10).

Wild mustard seed germination rate was affected by light, priming, and the interactive effect of priming × light × smoke type (P<0.001). The 50% of total germination was achieved only in seeds that were primed with 1/1 v/v and/or 1/10 v/v dilutions of both smoke types under both light regimes (Figure 3.9 & Table 3.4). In both light regimes, germination was faster (lower T_{50}) in seeds primed in 1/1 v/v of alfalfa smoke compared to seeds primed in the 1/10 v/v dilution (Figure 3.9 A, B and Table 3.4). Similarly, seeds primed in a 1/1 v/v dilution of wheat smoke, germinated faster in 24 h darkness (Figure 3.9 C and Table 3.4). However, considering the alfalfa smoke solution, seeds primed in the 1/10 v/v dilution germinated faster in 24 h darkness while seeds primed in the 1/1 v/v dilution germinated faster under diurnal light, indicating that the seed germination speed (T_{50}) varies with smoke dilution and incubating light condition.

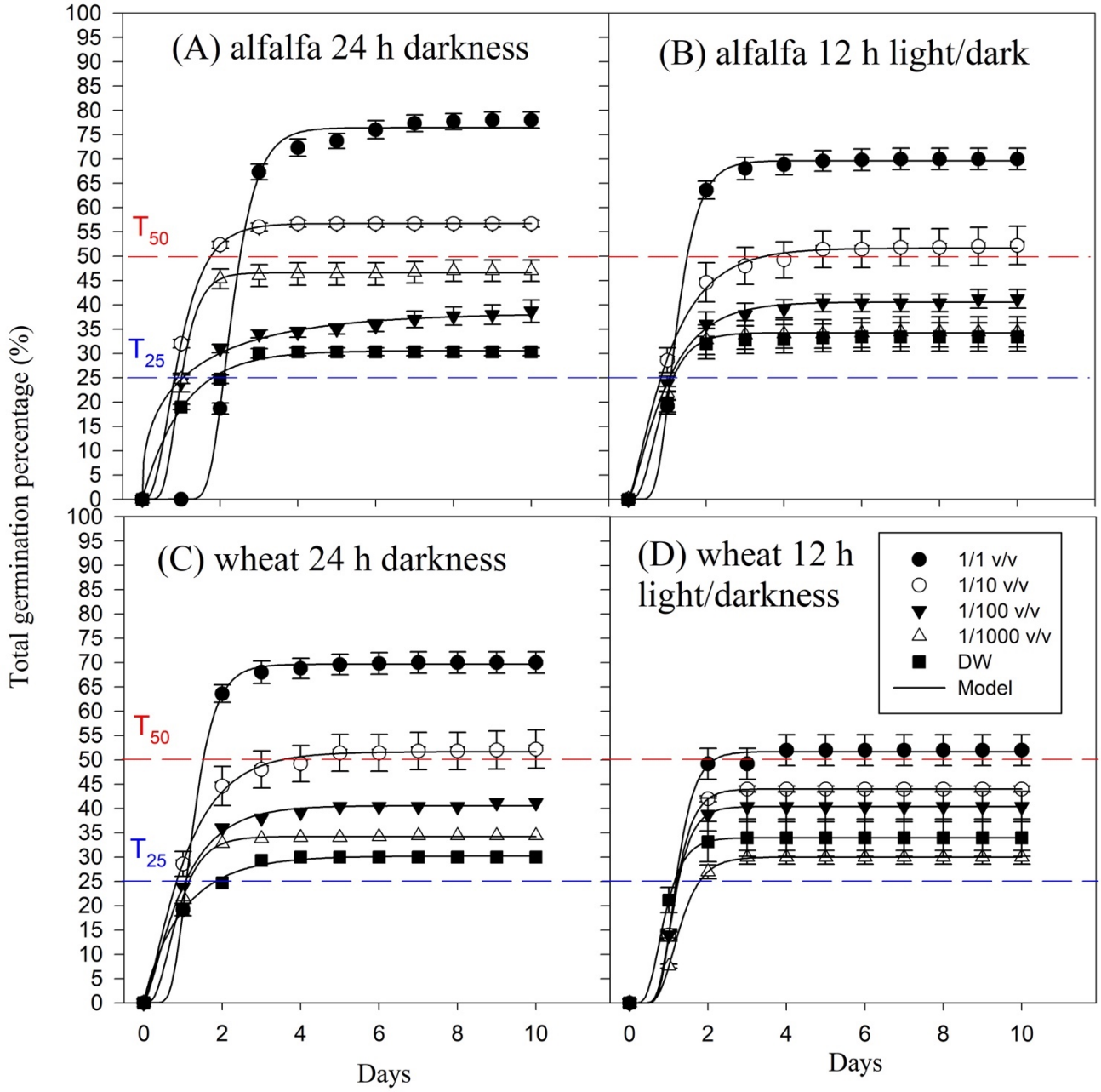


Figure 3.9 Germination curves of wild mustard (*S. arvensis*) seeds showing differing germination rates after priming in serial dilutions of aqueous smoke solution and incubation light regimes. (A) alfalfa, 24 h dark (B) alfalfa, 12 h light/dark (C) wheat, 24 h dark (D) wheat, 12 h light/dark, and incubated at 20 °C (DW: HPLC grade distilled water as the control. Black solid lines represent the predicted germination time courses within each smoke type-light regime fitted using a three-parameter Chapman-Richard's function (Eq. 3.1). Blue and red dashed lines identify 25% (T_{25}) & 50% total germination (T_{50}), respectively. (means ± S.E., n = 5 or 10).

As the germination rate estimates of T_{25}, seeds primed with alfalfa dilutions of 1/1000, 1/100, and 1/10 v/v showed significantly faster germination (lower T_{25}) ($P<0.05$) compared to the distilled water control in 24 h darkness (Figure 3.10). Wild mustard seed germination speed (T_{25}) was independent of smoke type (Appendix Table A.3.7). Seeds primed in 1/1 v/v dilution showed the slowest germination; albeit this achieved the highest germination. However, during the 12 h light/dark condition, only the seeds primed with 1/10 v/v dilution of alfalfa smoke, showed a faster germination relative to the control. Wild mustard seeds germinated much more quickly ($P<0.05$) under diurnal light than in 24 h darkness after seeds primed in the 1/1 v/v dilution of alfalfa smoke solution.

Interestingly, wild mustard seeds primed with all dilutions of wheat-derived smoke, germinated more quickly (low T_{25}) under 24 h darkness ($P<0.05$) compared to the control (Figure 3.10). However, under diurnal light condition, no seeds primed in wheat-derived smoke, germinated more quickly compared to the control. Also, the seed germination speed was similar between 24 h darkness and diurnal light conditions in all smoke dilutions.

Table 3.4 Days to 50% total germination (T_{50}) for wild mustard (*S. arvensis*) seeds after priming in serial dilutions of aqueous smoke solutions made from alfalfa hay (n = 10) or wheat straw (n = 5) and incubation at 20 °C in 24 h darkness (D) or 12h light / dark alteration (L/D). (DW: HPLC grade distilled water as the control. (DW: HPLC grade distilled water as the control. (means ± S.E., n = 5 or 10).

Smoke type		T_{50} (days) with smoke concentration effect				
		DW	1/1000 v/v	1/100 v/v	1/10 v/v	1/1 v/v
Alfalfa hay	T_{50} -D	-	-	-	1.78 ± 0.1^{aB}	2.5 ± 0.0^{aA}
	T_{50} -L/D	-	-	-	3.50 ± 0.1^{aA}	1.5 ± 0.0^{bB}
Wheat straw	T_{50} -D	-	-	-	3.40 ± 0.1^{a}	1.7 ± 0.1^{bA}
	T_{50} -L/D	-	-	-	-	2.2 ± 0.1^{aA}

D; 24 h dark incubation, L/D; 12 h light /dark alternation "-" represents primed seeds that did not achieve 50% final germination (T_{50})

a, b = Means within a row with the same lower-case letter are not significantly different ($P>0.05$).

A, B = For a given smoke type, means within a column (between D & L/D) with the same upper-case letter are not significantly different ($P>0.05$).

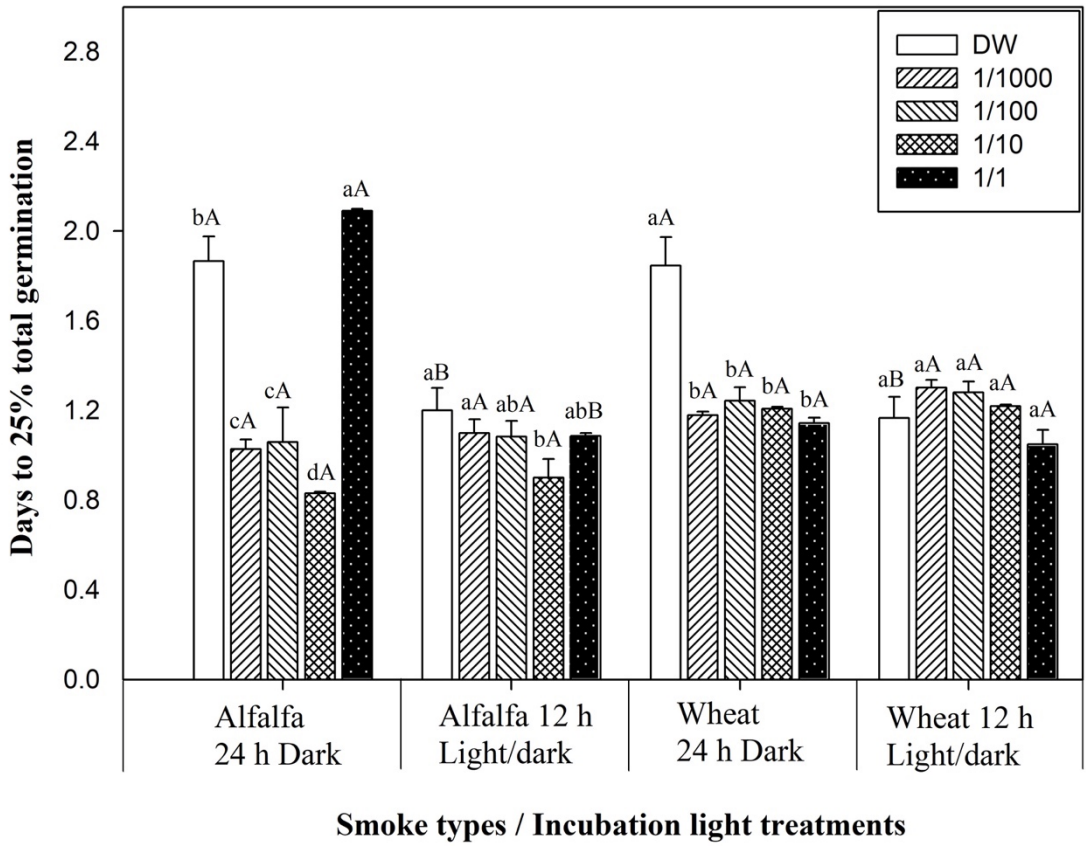


Figure 3.10 Days to 25% total germination (T_{25}) for wild mustard (*S. arvensis*) seeds after priming in serial dilutions of aqueous smoke solutions made from alfalfa hay (n = 10) or wheat straw (n = 5) and incubation at 20 °C in 24 h darkness or 12h light / dark alteration. (DW: HPLC grade distilled water as the control. Means with different letters were significantly different ($P \leq 0.05$) within each of four smoke type-light combination. (means ± S.E., n = 5 or 10).

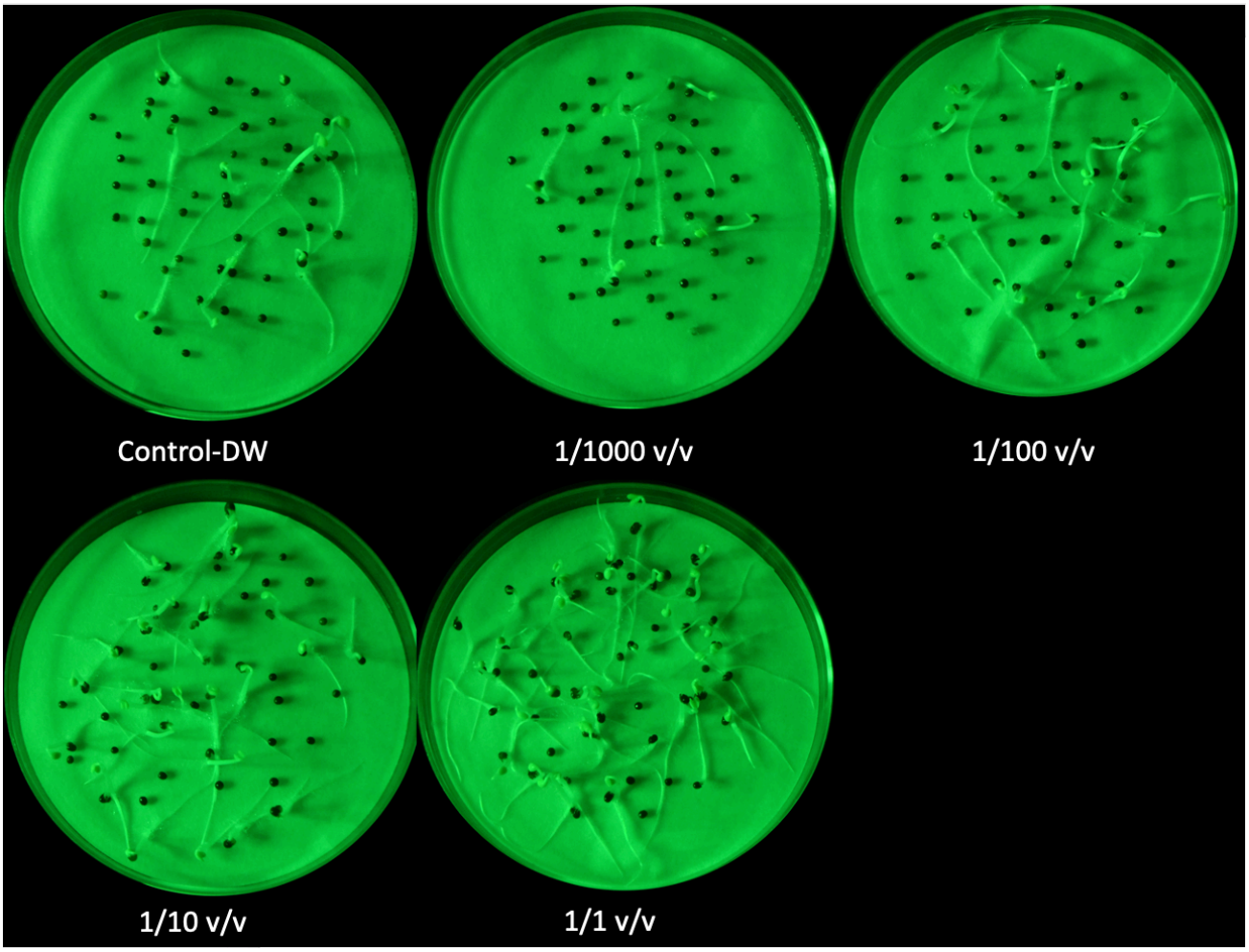


Figure 3.11 Germinating wild mustard (*S. arvensis*) seeds primed with distilled water (DW) or different dilutions of alfalfa aqueous smoke solutions on the 4th day of incubation at 20 °C in 24 h darkness.

3.4 Discussion

3.4.1 Comparative effect of smoke solutions derived from different plant species on seed germination percentage and rate

High concentrations of smoke water, such as 1/1 and for some plant materials 1/10 v/v dilutions, reduced both germination percentage and germination rate of lettuce seeds compared to the distilled water control, suggesting the presence of inhibiting or toxic compounds activity at high concentrations (Dixon et al., 1995; Clarke & French, 2005). This observation agrees with Kulkarni et al. (2007), where *L. sativa* and *Acacia robusta* exhibited a poor germination performance after being treating with highly concentrated smoke solutions. It was found that these inhibitory effects might be avoided by first imbibing seeds on concentrated smoke solution for a short period followed by transferring seeds to distilled water (Brown, 1993b). A germination inhibiting compound, trimethylbutenolide [TMB; 3,4,5-trimethylfuran-2(5H)-one], was isolated from the smoke water, and showed the ability to impair *L. sativa* seed germination responses (Light et al., 2010). Its activity was also minimized with increasing dilution of TMB compound (Light et al., 2010). Under natural conditions, these inhibitory toxic compounds might be washed away or diluted after rain providing a mechanism to stop seeds germination until the condition is suitable (De Lange & Boucher, 1993; Light et al., 2002; Sparg et al., 2005). Smoke from alfalfa, wheat, *Eragrostis,* and *Melinis,* slightly enhanced seed germination even at high concentration (1/10 v/v) only at alternating 25/15 °C, darkness (Figure 3.4 a). This indicates that a smoke concentration effect also depends on smoke type (plant species used to prepare smoke solutions) and growth conditions.

The 1/1000 v/v concentration showed the most significant enhancing effect on seed germination among all smoke types in darkness (Figure 3.4 a). This finding agrees with a study that showed diluted smoke solution treatments increased seed germination of photoblastic seeds of *Apium graveolens* (Thomas & van Staden, 1995). Regardless of the origin or locality of those plant materials used to produce smoke solutions, both 1/1000 and 1/100 v/v dilutions of alfalfa and wheat improved seed germination similar to South African smoke solutions. This suggests that seed germination promotive activity does not depend on the ecological region of plant materials used for smoke solution generation (Jäger et al., 1996a; Çatav et al., 2012).

In this study, seed germination rate was not significantly improved in a constant 25 °C after smoke water treatment except with 1/1000 v/v dilution of wheat smoke. A similar observation was

reported for 19 Mediterranean woody and herbaceous species, which showed slow germination speed after smoke water treatments (Reyes & Trabaud, 2009; Çatav et al., 2012).

3.4.2 Seed germination responses elicited by smoke solutions are temperature, light, and smoke type dependent

A constant 25 °C temperature has been widely used as the optimum germination temperature for *L. sativa* (var. Grand Rapids) during bioassay testing (Drewes et al., 1995; Flematti et al., 2004; van Staden et al., 2004). Abu et al. (2016) used 25/15 °C incubation temperatures to test the effect of smoke solutions on lettuce seeds in comparison with forage species. In this study, I used both 25 °C and 25/15 °C, which revealed an interactive response between smoke types and germination conditions. My result is consistent with Ren & Bai (2016a) that various dilutions of alfalfa smoke solutions did not affect seed germination of Salad Bowl lettuce at 25 °C in darkness. However, the 1/1000 v/v smoke solution derived from two graminoids (wheat and *Eragrostis*) increased the seed germination percentage and rate compared to control at 25 °C in darkness. In another word, all smoke types tested in this study have the capability to enhance seed germination at alternating temperatures, but only two graminoids (wheat and *Eragrostis*) at constant temperature. This suggests that smoke derived from graminoids may have different active chemicals than that from forbs/legumes. So far, in most research attempts, active chemicals have been isolated from smoke derived from plants that belong to graminoids (Flematti et al., 2004; van Staden et al., 2004). Considering germination responses in this and other studies, alternating temperatures are recommended for further studies of smoke-elicited seed germination mechanisms.

On the contrary, seed germinated fast only at constant 25 °C after treating lettuce seeds with 1/1000 v/v dilution of wheat smoke solution. However, comparatively alfalfa or wheat smoke solution treated lettuce seeds germinated faster at alternate 25/15 °C, darkness condition than that of at 25 °C, darkness in this study. This is consistent with the former research finding that lettuce seeds germinated faster at 25/15 ˚C alternating temperature and darkness after smoke solution priming (Abu et al., 2016). It was evident that seed germination speed interacts with ambient environmental conditions such as temperature and light regimes. The seed germination rate of different species responds differently to smoke solutions prepared from different plant materials (Çatav et al., 2012).

Borthwick et al. (1952) observed that *L. sativa* (lettuce) seed germination is induced by light. Seeds with the requirement of light for germination are termed photodormant or light-sensitive

seeds. Plant-derived smoke solutions can substitute this light requirement of Grand Rapids lettuce seeds germination (Drewes et al., 1995). In this study, diluted smoke solution generated from all plant materials significantly improved seed germination compared to distilled water control in darkness. This indicates that all smoke types tested have chemical compound/s with the ability to change lettuce seeds' phytochrome system in a way that promotes seed germination. This observation is in agreement with previous work on smoke-elicited seed germination of light-sensitive seeds, including *L. sativa, Apium graveolens, Shoenia filifolia* subsp. *Subulifolia, Conyza canadensis* in darkness (Drewes et al., 1995; Thomas & van Staden, 1995; Plummer et al., 2001; Ren & Bai, 2016a).

Smoke-treated seeds germinated faster than distilled water control only in darkness but not in the light, suggesting that the germination speed also includes within the mechanism of smoke-elicited seed germination. However, smoke-treated seeds germinated faster at an alternative 25/15 °C than that of at constant 25 °C in darkness. This agrees with the former study that the effects of smoke treatments on seed germination rate are temperature dependent (Ghebrehiwot et al., 2009).

3.4.3 Seeds from different fire-ephemeral species responds differently after smoke solution priming treatment

This is the first study that reports the smoke-stimulated germination responses of these fire ephemerals except *E. curvula*. Interestingly, this study demonstrates the ability of smoke in stimulating seed germination of species that shows a wide diversity of plant characteristics, for example, life forms (forbs, graminoids), fruit and seed types, etc. In this study, different radicle lengths as the germination criteria were selected from previous, species-specific reports and also based on different seed sizes of species. Higher radicle lengths (≥2 mm) have been taken for larger-sized seeds as the germination criteria whereas lower radicle lengths (≥1 mm) for smaller-sized seeds (Steiner et al., 2019).

E. curvula seeds did not show a significant difference between light and dark conditions on both germination and germination rate, suggesting that light condition is not a limiting factor on smoke-elicited seed germination of this species. This finding agrees with the previous work, where percent germination and shoot and root length of *E. curvula* was increased by smoke water treatment (Clarke & French, 2005; Ghebrehiwot et al., 2008). However, in their study, smoke solution was prepared by burning the *Themeda triandra* Forssk. grass species or a mixture of plant species,

including graminoids and forbs. This species showed the highest germination percentage among other species tested. Clarke & French (2005) reported the additive effect of both smoke and heat on increasing germination percentage in *E. curvula* when applying both the smoke and heat together. In addition, other *Eragrostis* species such as *E. leptostachya* and *E. benthamii* (Clarke & French, 2005) have been shown smoke-stimulated seed germination. Germination rate was increased in both light conditions. On the contrary, seeds of this species primed in smoke derived from grasses or butenolide could not improve its germination rate (Ghebrehiwot, 2010).

The other graminoid species, *C. pospischilii,* showed smoke-stimulated seed germination after priming with the lowest concentration of alfalfa smoke solution in both darkness and light conditions. This is the only other species in addition to *E. curvula* that responded positively to smoke under both light regimes. This finding agrees with Williams et al. (2014), in which they reported significant smoke-stimulated germination of other species of this genus, *Cymbopogon obtectus* in darkness. However, other species such as *C. refractus* did not show a germination improvement with smoke treatment under light conditions (Read & Bellairs, 1999; Clarke & French, 2005). Similarly, *M. nerviglumis* did not show smoke-stimulated seed germination under diurnal light conditions, whereas under darkness, diluted smoke concentration increased the *M. nerviglumis* seed germination. This result is in agreement with a former work where the invasive species, *Melinis minutiflora* did not affect by either smoke or heat treatments on seed germination and rate under light/dark light regime (Gorgone-Barbosa et al., 2020)

Germination of *I. hilaris* seeds primed in smoke solutions was low but was higher when germinated in darkness. Similarly, a low but non-significance effect of smoke on germination of other *Indigofera* species (*I. australis*) was reported (Clarke et al., 2000; Brown et al., 2003, 2004). In those studies, they have been used a mixture of native vegetation to produce smoke solutions. However, heat treatment can break seed dormancy of *Indigofera* species by about 45% (Clarke et al., 2000).

Light is required for dormancy breaking in germinating *Helichrysum* species (Willis & Groves, 1991; Afolayan et al., 1997), and gibberellic acids under light further increased seed germination (Bunker, 1994; Afolayan et al., 1997). In this study, germination was improved significantly by smoke in darkness, agreeing with previous reports where several other *Helichrysum* species were stimulated after smoke treatments (Brown et al., 1993a, 2003, 2004). On the contrary, seed germination of *Helichrysum aureonitens* was inhibited by smoke extract prepared using South African fynbos vegetation (Afolayan et al., 1997).

Seed germination of *H. capensis* was enhanced by smoke treatment in the presence of light only, similar to the response of a similar species, *Vernonia natalensis* (Ghebrehiwot, 2010). Germination of another similar species, *Vernonia saligana*, was inhibited by smoke in darkness (Liang et al., 2020).

3.4.4 Possibility of smoke-aided weed control of wild mustard and its use as a novel bioassay species

There is growing evidence that plant-derived smoke solutions stimulate seed germination of a range of weed species, triggering synchronous germination from dormant weed seedbanks (including herbicide-resistant weeds) (Adkins & Peters, 2001; Daws et al., 2007; Steven et al., 2007; Long et al., 2010; Mojzes et al., 2015). In particular, weed seeds of the family Brassicaceae are well known for consistent, inherent smoke responsiveness and smoke-stimulated seed germination (Long et al., 2011; Godakanda et al., 2020).

Wild mustard (*S. arvensis*) exhibited low germination percentage both in darkness (30 ± 0.8%) and light (34 ± 2.8%). The seed coat-imposed dormancy and insensitivity to light in this species can be overcome by priming (Chacur & Takaki, 1996), similar to radish seeds (Gonçalves & Takaki, 1997).

Most weedy species require light to break dormancy and/or alternating temperature to break dormancy (Baskin & Baskin, 1998; Daws et al., 2002; Batlla & Benech-Arnold, 2010). In a greenhouse study, Goudey et al. (1987) found rather than a single treatment, a combination of light, KNO_3, and NH_4Cl at 20 °C, stimulated the seed germination over 90% in dormant wild mustard seeds. This indicates wild mustard seeds can germinate after tillage and the application of nitrogen fertilizers.

My results indicate that wild mustard seed germination and rate were significantly improved under both darkness and light incubation after either alfalfa or wheat smoke water priming, particularly with a very high concentration smoke solution. Similar results were reported for *Conyza canadensis* (Ren & Bai, 2016a), *Brassica tournefortii* (wild turnip), and *Raphanus raphanistrum* (wild radish) (Stevens et al., 2007), suggesting that these species require highly concentrated stimuli to break dormancy.

However, after one day of incubation, the highest smoke concentration (1/1 v/v) stimulated none of the seeds to germinate, while the seeds primed in 1/10 v/v dilution increased germination

significantly (by 71%). From the third day of incubation onwards, the highest germination was observed in wild mustard seeds primed in the highest dilution (1/1 v/v) of both alfalfa and wheat smoke under both light regimes. This indicates that the concentrated smoke dilution (1/1 v/v) takes an additional 1–2 days compared to other smoke dilutions to activate the physiological mechanisms inside seeds that provide the highest germination percentage. In addition, the non-significant germination was evident in seeds primed with 1/100 v/v dilution of alfalfa smoke only under 24 h darkness incubation (Figure 3.7). This was observed throughout the testing period (Figure 3.8). The absence of promotive activity of this 1/100 v/v alfalfa dilution has less biological meaning since the general trend was that the germination was improved with increasing smoke concentration.

My results confirmed that smoke treatment alone can be successful in stimulating germination of wild mustard seeds in darkness in contrast to previous reports (Adkins & Peter, 2001). Daws et al. (2007) reported that the presence of toxic or inhibiting compounds in crude smoke solution, compared to isolated butenolide solution, might be the reason for the negative or neutral effects of smoke on seed germination percentage, rate, and seedling growth of *Sinapis alba* L. However, smoke isolated butenolide and GA_3 increased the germination of this species significantly at 15 °C in light/dark conditions. Other weedy species in the Brassicaceae family, such as *Brassica tournefortii* (wild turnip), *Raphanus raphanistrum* (wild radish), *Sisymbrium orientale, Capsella bursapastoris*, has significantly stimulated seed germination after smoke or/and butenolide application (Daws et al., 2007; Stevens et al., 2007; Dixon et al., 2009; Long et al., 2011) suggesting the inherent smoke responsiveness of members of this family.

The rapid germination of wild mustard seeds within one to three days after treatment with smoke solutions prepared either from graminoid (wheat) or forbs (alfalfa) makes this species a useful, rapid, broad, and simple bioassay system to detect the bioactivity of different types of smoke solutions (Light*,* 2006). It is worth noting that this species is stimulated even by the highest smoke concentration, which is useful in chemical isolation process of active compounds. This is because, in previous chemical isolation processes, bioactivity was observed mostly with a diluted fraction of smoke. Therefore, it was necessary to concentrate this diluted fraction using a variety of separating techniques to increase the detection level of active compounds with existing methods and thereby recommend some optimized separation protocols (Flematti et al., 2008). In contrast, with wild mustard seeds, it's possible to use a smoke solution at its highest concentration for further chemical analysis where the sensitivity of chemical detection techniques improves with an increasing concentration of analytes of interest. Furthermore, germination of wild mustard seeds showed smoke

concentration dependence over a broad range of doses (i.e., thousand-fold). Reproducible results over two repeats in my trials highlight the suitability of this species as an alternative bioassay in future research.

If laboratory findings are validated under field conditions, application of alfalfa/wheat smoke solutions onto croplands after harvest at the end of summer/fall might stimulate the dormant seed bank and synchronize the germination of weedy wild mustard. Similar approaches have been proposed with other weeds (Kamran et al., 2014; Mojzes & Kalapos, 2016). In Western Australia, the period just before the cropping season was found to be the best time to apply KAR1 (one of the active compounds in smoke) to trigger the germination of weedy species (Long et al., 2010). After stimulating profuse germination of dormant weedy species, a secondary chemical control method such as herbicide/weedicide treatment can be applied with great effect, minimizing overall chemical use (Adkins & Peter, 2001; Dixon et al., 2009; Ghebrehiwot, 2010; Kamran et al., 2014). Current weed management practices in herbicide target only about 9% of germinable weed seeds during the growing season, missing the majority (~90%) of the weed seed bank that is dormant in the soil (Stevens et al., 2007). Removal of weed seed germinants from the field before the next crop cycle will reduce the germination of weedy species and thereby reduce the seed inputs into the persistent seed bank (Mojzes & Kalapos, 2016). This will help the depletion of the major agricultural weed soil seed banks within a minimum growing cycle (Boutsalis & Powles, 1998).

3.5 Conclusions

Smoke-promoted lettuce seed germination is only evident in dark conditions with diluted smoke solutions of all smoke types. Similarly, germination speed of lettuce seeds treated with alfalfa or wheat smoke solution, was enhanced at 25/15 °C under darkness. Consistent with my second hypothesis, at 25 °C in darkness, only two graminoid-based smoke types (wheat and *Eragrostis*) produced smoke enhanced seed germination. But all smoke types were effective at alternating 25/15 °C darkness. Alternating temperatures are recommended for further studies of smoke-elicited seed germination mechanisms with the lettuce model plant. Contrary to my first hypothesis, smoke-elicited seed germination is independent of the ecological region of different plant materials which were used to prepare smoke types. Consistent with the third hypothesis, though, alfalfa or wheat smoke solution stimulates germination not only lettuce seeds but also in fire ephemeral, South African seeds (except *H. capensis*), and wild mustard seeds, which belong to major smoke-

responsive plant families. Wild mustard seeds showed promising characteristics that make it suitable as a bioassay species to detect plant-derived smoke chemical compounds. My findings suggest that alfalfa/wheat smoke solution has wide applicability as a weed seed germination stimulant with special reference to wild mustard seeds. Further, I suggest that smoke solutions, as a potential weed management tool, be integrated with other existing weed management practices.

CHAPTER 4. EXTRACTION, FRACTIONATION AND PREDICTION OF POSSIBLE BIO-ACTIVE COMPOUNDS PRESENT IN ALFALFA SMOKE USING LETTUCE SEED BIOASSAY GERMINATION

4.1 Introduction

Fire is a natural disturbance and selective force in many ecosystems (Keeley et al., 2012) and plays a key role in maintaining ecosystem structure, function, and diversity (Bond & Keeley, 2005; Keeley & Brennan, 2012). The heat from a fire can break seed dormancy and assist in seedling recruitment (Keeley & Fotheringham, 2000; Dixon & Barrett, 2003; Reyes & Trabaud, 2009). After a fire, reduced canopy cover can modify light quality and quantity (Light, 2006). Fire also alters moisture and nutrient conditions, and compounds produced by fire that are found in smoke can enhance seed germination (van Staden et al., 2000). Perhaps because of these effects, plants in fire-prone ecosystems have adaptations to fire-related factors (Everson & Tainton, 1984; Everson et al., 1988; Bond & van Wilgen, 1996). Interruption of seed dormancy can be induced by heat-shock or heat-induced scarification to break hard seed coats (Musil & de Witt, 1991; Brits et al., 1993; González-rabanal & Casal., 1995). Direct embryo stimulation followed by embryo imbibition (Martin et al., 1975; Baskin & Baskin, 2014) can also occur.

Chemical cues from ethylene and ammonia (Van de Venter & Esterhuizen, 1988), nitrogen oxides (Keeley & Fotheringham, 1997), ash (Henig-Sever et al., 1996), and smoke (De Lange & Boucher, 1990; Brown, 1993a) can stimulate seed germination. About 57% of species belonging to 139 families in Mediterranean ecosystems in Europe and North America (Pausas & Keeley, 2014), 54% of South African plant species (Brown et al., 1993; Brown & van Staden, 1997), and 45 species of native Western Australian plants (Dixon et al., 1995) have responded positively to smoke or smoke extracts. Smoke contains thousands of chemical compounds (Smith et al., 2003), and it is likely that these compounds from different plant species have unique characteristics that affect seed germination in multiple ways (Ren & Bai, 2016a).

However, only two studies have differentiated the effects of smoke based on the plant material from which it was derived. The smoke produced during the burning of leguminous plant materials was found to promote seed germination percentage, germination rate and/or seedling length (Kamran et al., 2014; Ren & Bai, 2016a). Several active compounds from plant-derived smoke have been identified, including karrikin (KAR1) or 3-methyl-2H-furo [2,3-c]-pyran-2-one (Flematti et al., 2004; van Staden et al., 2004), cyanohydrin glyceronitrile (Flematti et al., 2011a),

hydroquinone (1,4-benzenediol) (Kamran et al., 2017) and catechol (1,2-benzenediol) (Wang et al., 2017). Among these, KAR1 was the most striking as it induced a significant increase in germination or/and seedling vigor of diverse plant families/species, including crops (Kulkarni et al., 2006; van Staden et al., 2006; Jain et al., 2008), fire-prone plants (Merritt et al., 2006), and arable weeds (Daws et al., 2007; Stevens et al., 2007). Ren et al. (2017) analyzed the chemical profile of smoke solutions derived from a legume species but failed to detect KAR1, suggesting that compounds other than KAR1 might be responsible for promoting seed germination.

Analysis of chemically complex smoke solutions is difficult, but bioassay-guided fractionation processes have been adopted for the detection of active compounds (Drewes et al., 1995; Flematti et al., 2008). In classical liquid-liquid partitioning, active fractions are separated based on chemical compound polarity. Each resulting fraction is comparatively less complex than smoke water. The unwanted complex chemical matrix effect of smoke water is thereby reduced by fractionation (Flematti et al., 2004; van Staden et al., 2004; Kamran et al., 2017; Ren et al., 2017). Methods based on GC-MS and HPLC-DAD-MS have been adopted for routine identification of compounds in smoke (Light, 2006; Flematti et al., 2008; Wang et al., 2017). NIST maintains a mass spectral library with over two million fingerprints from over 350,000 known compounds (Mak, 2020). Comparisons between experimentally obtained mass spectra of unknown compounds and this library provide insights for accurate and rapid identification of chemical species (Simon-Manso et al., 2013; William, 2019).

A macroporous resin, such as non-polar FPX-66, has been widely used to separate and isolate low molecular weight plant metabolites from complex plant extracts (Liu et al., 2013; Torres et al., 2014; Sandhu & Gu, 2013). Non-polar FPX-66 resin absorbs non-polar compounds such as organic polysaccharides, acidic compounds, etc. If resins were used, during adsorption from a polar phase, compounds (adsorbates) in smoke water diffuse into solid resin particles (adsorbent), where the concentration of active compounds increases through molecular interactions (Kammerer et al., 2011; Petrotos et al., 2016). To my knowledge, there is no published study on the use of macroporous resin to extract active compounds in smoke water to study the effect of fractions in the Salad Bowl lettuce seed bioassay.

Alfalfa-derived smoke water has been shown to promote germination of selected fire-responsive Fescue Prairie forbs and substituted for the light requirement for germination. However, no KAR1 has been detected in alfalfa smoke (Ren, 2015). The main objective of my study was to isolate and identify chemical compounds that enhanced seed germination in the Salad Bowl lettuce

bioassay. I hypothesized that non-KAR1 chemical compounds in alfalfa smoke promote seed germination. I used a combination of extraction methods (resin and C18 RP-CC) and analytical techniques (HPLC, GC-MS and NMR).

4.2 Materials and Methods

4.2.1 Plant materials and chemicals/reagents

Seeds of the cultivar *Lactuca sativa* L. cv. Salad Bowl that showed a consistent germination response to alfalfa-derived smoke water (Chapter 2) was purchased from Early's Farm & Garden Centre in Saskatoon; SK. Seeds were stored at –20 °C until they were used.

Other materials for my study included resinous FPX-66 from Rohm and Haas Co. (Philadelphia, PA), Anhydrous ethanol from Commercial Alcohols Inc., Montréal, QC, Canada, Ethyl acetate (EtOAc), HPLC-DW, and LC-MS grade methanol from VWR International LLC., ON, Canada. Methanol-d_4 (CD_3OD), chloroform-*d* ($CDCl_3$) and deuterated water (D_2O), anhydrous sodium hydroxide (NaOH), acetonitrile (ACN), and anhydrous sodium sulfate (Na_2SO_4) were obtained from Sigma-Aldrich Co., St. Louis, MO, USA and a sample of pure KAR1 (0.1mg/ mL) was purchased from Toronto Research Chemicals Inc. Gibberellic acid (98% GA_3) was purchased from Thermo Fisher Scientific Inc.

4.2.2 Salad Bowl lettuce bioassay

In my study, all chemically separated fractions were tested for seed germination bioactivity using this lettuce seed bioassay. Five replicates of 30 seeds placed in 10 cm Petri dishes lined with two layers of Whatman #1 filter paper were used for each smoke water dilution in a completely randomized design (CRD). Control seeds were imbibed in 4 mL of HPLC-DW in the dark at 25/15 °C for one day (Conviron A1000 Plant Growth Chambers, Controlled Environments Limited). In each treatment, 4 mL of smoke solution was poured onto the filter paper containing lettuce seeds. All germination bioassays were conducted in the dark and observed using a “green safe” light (540 nm, 0.3–0.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Drewes et al., 1995; Kulkarni et al., 2007). The "safe green" light was produced by covering a general electrical GE 540G green, fluorescent bulb using two layers of green (Roscolux 95 1.8 mil) plastic light filters (Rosco Holdings, Inc.) (Jones et al., 2004). All Petri dishes were sealed in zip-lock bags to avoid water loss and covered with two layers of aluminum foil to

reduce light exposure. Radicle emergence was taken as the criteria for germination, which was recorded after one day. Experiments were terminated when no germination was observed for three consecutive days. Germination percentages after one day of incubation, hereafter referred to simply as germination, were plotted for each smoke type. One day of incubation was chosen because lettuce seed germinates rapidly after 24 h of exposure to smoke solution (Light, 2006; Ren, 2015).

In addition to the initial alfalfa smoke stock solution (1/1 v/v), the Salad Bowl lettuce bioassay experiment was conducted on resin separated fractions (1/1 v/v), the liquid-liquid partitioned fraction (1/1 v/v), and C18 purified fractions (1/1 v/v) of alfalfa smoke water. Three dilutions of 1/10 v/v, 1/100 v/v, and 1/1000 v/v were prepared from each type of smoke stock solution (1/1 v/v) to investigate concentration effects. To determine the comparative effects of KAR1 (Karrikin) and GA_3 (gibberellic acid) on bioassay germination, a concentration of 10^{-5} M of KAR1 and GA_3 were dissolved in distilled water to be used as the 1/1 v/v KAR1 and GA_3 solutions, respectively.

4.2.3 FPX-66 Resin fractionation of alfalfa smoke solution

The resin FPX-66 (Table 4.1) was chosen to separate seed germination promoting molecules from the alfalfa smoke extract into fractions of different polarity. The FPX-66 resin separation procedure for alfalfa smoke active fractions was based on methods developed by Zhang et al. (2019) and Padilla et al. (2018). During commercial resin production, undesired substances, such as monomers and porogenic agents, are trapped inside resin pores (Zhang et al., 2019), and those are removed by pretreatment. Resins were cleaned by soaking in two bed volumes (BV) of 96% ethanol overnight and then washing with 5 BV of distilled water before use to completely remove ethanol (Padilla et al., 2018).

Table 4.1 Physical properties of adsorbent resin, FXP-66.

Resin	Chemical nature	Polarity	Surface area (m^2/g)	Average pore diameter (Å)
FXP-66	polystyrene−DVB [a]	non-polar	700	200–250

DVB [a]; divinylbenzene (Sandhu & Gu, 2013)

Forty-nine grams of FPX-66 resin (dry weight) was introduced into 1 L of alfalfa aqueous smoke solution (pH 7.43) in a 2 L volumetric flask at 25 °C (Figure 4.1). The flask was shaken (120 rpm) in an orbital shaker (Orbital Shaker, VWR International, Radnor, PA, USA) for 2 hours at room temperature (22.5 °C). After adsorption, resins were filtered and washed with 1 L of 10% ethanol/water (EtOH/H_2O). A polar aqueous phase (1 L) and 10% EtOH/water eluate (1 L) were collected for further bioassay. Their vacuum evaporated masses were 1.20 g and 0.70 g, respectively. Fractions were subject to NMR analyses. Resin beads collected after the 10% EtOH/water wash were again washed with 1 L of 100% EtOH. Filtrate collected from this final 100% EtOH wash was also used for lettuce seed bioassay and a vacuum evaporated sample (0.5 g) was used in NMR analysis (see Section 4.2.7 for details). To determine and eliminate the effects of ethanol on seed germination, 10% and 100% (v/v) ethanol was used as the solvent control in lettuce seed bioassay.

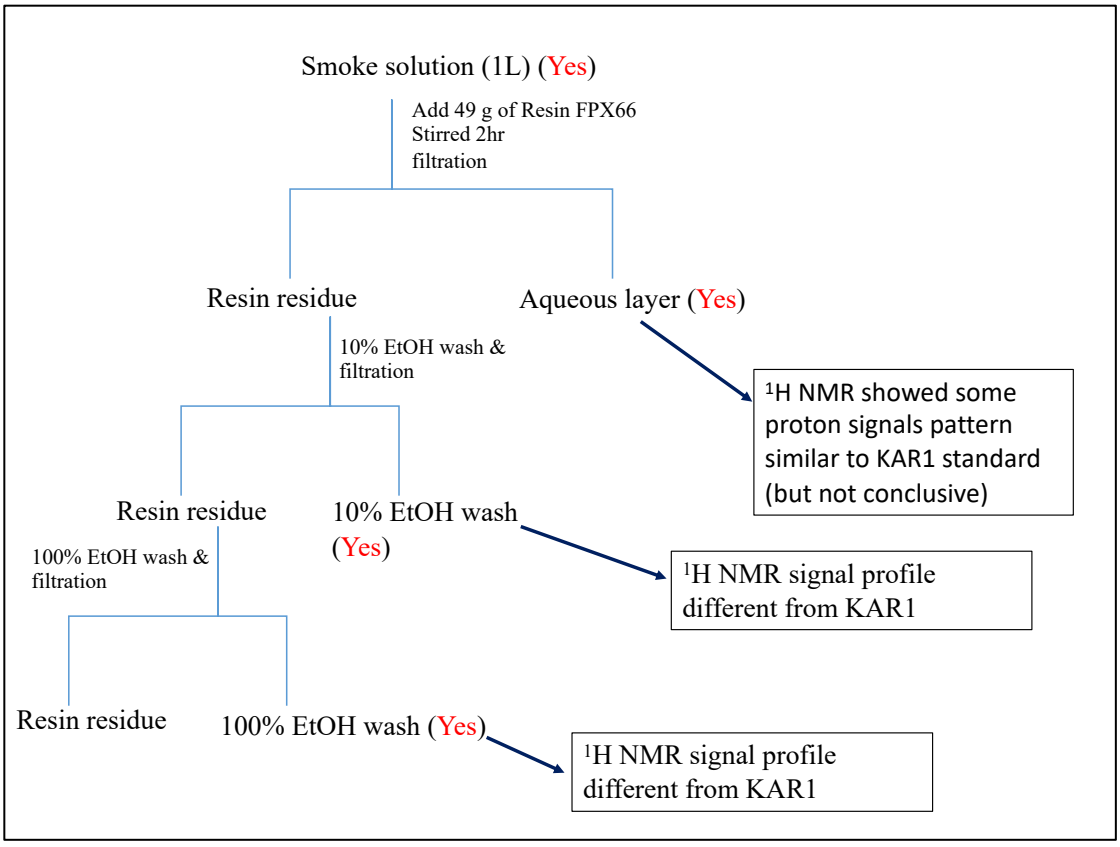


Figure 4.1 Flow chart of the resin fractionation method used with alfalfa smoke solution. ('Yes' indicates the promotive action showed on bioassay seed germination).

4.2.4 Liquid-liquid extractions of alfalfa smoke water

The smoke water active fraction separating method and HPLC analysis were based on Flematti et al. (2008) and Ren et al. (2017) with modifications. In total, 2 L of the stock smoke solution produced from alfalfa (pH 7.43) was filtered (32 cm, Whatman #1 filter papers) and separated. Each liter of the filtered smoke solution was completely extracted using EtOAc (3 × 200 mL) in order to separate the water and organic (EtOAc) fractions. The combined organic fraction was then washed five times with 1% (w/v) aqueous sodium hydroxide (NaOH) to remove the acid compounds resulting in an acidic and a neutral portion of smoke water. Water, organic, acidic, and neutral phases/fractions were diluted separately to make three serial dilutions: 1/1000 v/v, 1/100 v/v, and 1/10 v/v. Lettuce seeds were treated with each serial dilution, or HPLC-DW (0/1 v/v, as the control) in the Salad Bowl lettuce bioassay experiment (see section 4.2.2.).

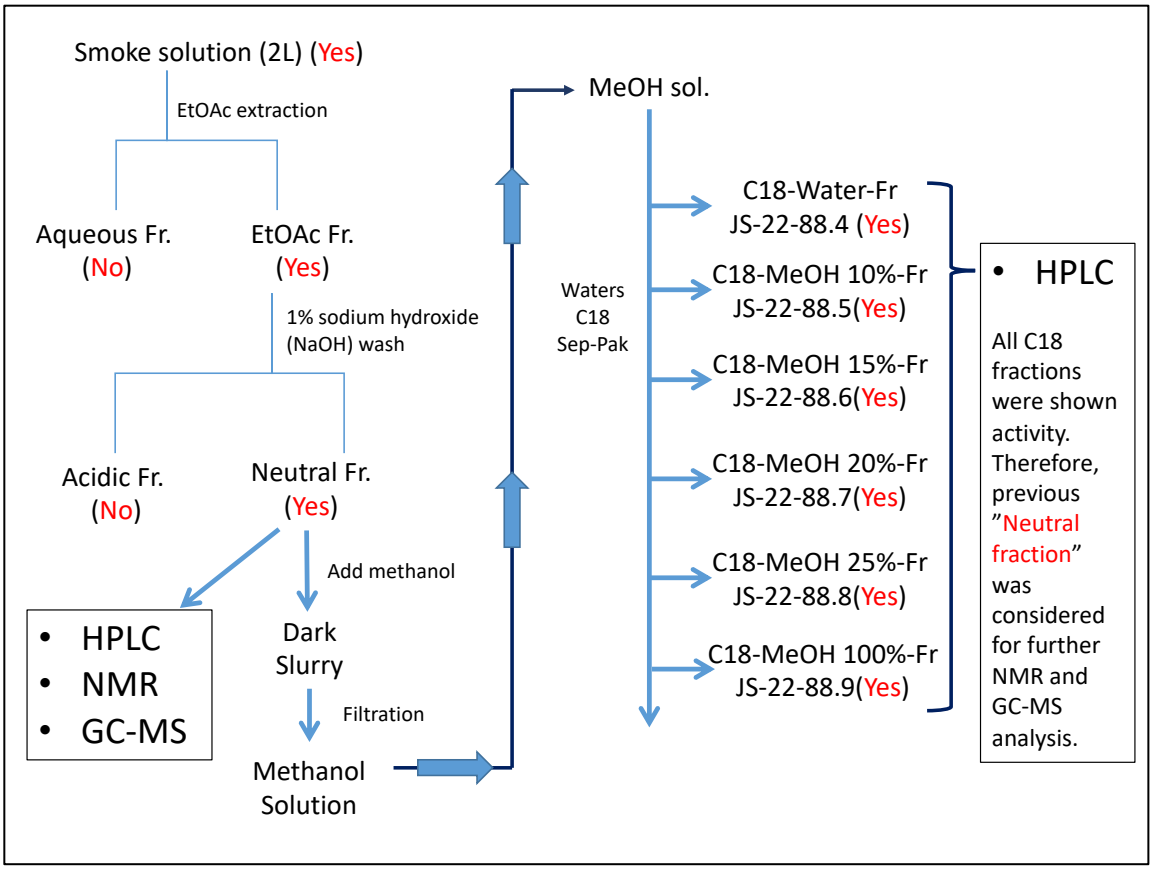


Figure 4.2 Flow chart of the liquid-liquid partitioning and C18 reverse-phase column chromatography method used with alfalfa smoke solution and relevant layers taken for HPLC, NMR and GC-MS analysis. ('Yes' indicates promotive action on seed germination).

4.2.5 C18 Reverse phase Column chromatography (C18 RP-CC)

The bioactive neutral fraction collected at the end of the liquid-liquid extraction in section 4.2.4 was dried with 90 g of anhydrous sodium sulfate (Na_2SO_4), filtered and evaporated in a vacuum to produce neutral fractions (103 mg from alfalfa). Half of concentrated neutral fractions (~55 mg) were then subjected to reversed phase (RP) tC18 column (Waters Sep-pak vac 12 cc cartridge, 2g), and eluted with a water: methanol gradient (methanol proportion: 0%, 10%, 15%, 20%, 25%, and 100% (v/v); 50 mL aliquots of each mixture) (right side of Figure 4.2). The six fractions (each 50 mL) collected in reverse phase column chromatography (RP-CC) were 20 times concentrated then compared to the initial 2L, 1/1 v/v alfalfa stock solution. Five milliliters of each fraction dissolved in 95 mL of distilled water was equivalent to the concentration of initial 1/1 v/v alfalfa stock solution. Those diluted solutions were used for the Salad Bowl lettuce bioassay test in darkness for 24 h to assess fraction bioactivity. To determine and eliminate the effects of methanol on seed germination, respective methanol proportion solutions (v/v) were used as the solvent control under each separated C18 fraction lettuce seed bioassay.

4.2.6 High-performance liquid chromatography (HPLC) analysis

For this analysis, only the active fractions of seed germination promoters, EtOAc separated neutral fraction (section 4.2.4) and C18 fractions (section 4.2.5), were used. These solutions were first evaporated to 1.5 mL under a vacuum. A portion of the vacuum evaporated active fraction (20 µL) from each was analyzed with a C18-RP HPLC column (Chromolith ® Performance RP-18e 100-4.6), eluted with acetonitrile; water gradient (7%-14%-95%-7%-7% acetonitrile/water over 0-14-15-16-20 min at 2 mL/min) for further fractionation. Detector wavelength (UV absorbance) for Karrikin (KAR1) measurement was set at 330 nm when an acetonitrile: water gradient was used (Flematti et al., 2008; Ren et al., 2017). A sample of 20 µL of pure standard KAR1 (0.1mg/ mL) (Toronto Research Chemicals Inc.) was eluted with the same acetonitrile-based method.

As all C18 separated fractions showed promotive activity for lettuce seed bioassay germination, further analytical testing (NMR and GC-MS) was done using the combined EtOAc neutral fraction (Section 4.2.7 and 4.2.8).

4.2.7 Nuclear magnetic resonance analysis

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance spectrometer (AV600) at 600 MHz for ^{1}H-NMR and 150.9 MHz for ^{13}C-NMR using methanol-d_4 (CD_3OD), chloroform- d ($CDCl_3$) and deuterated water (D_2O) as the solvent (Figure 4.3; Inverse triple resonance probe, TXI, 5 mm; Bruker-Topspin 3.6.1 software, 2018 Bruker BioSpin GmbH).

Active resin-separated fractions (4.1.3) and EtOAc neutral fraction were evaporated to dryness and reconstituted with excessive D_2O or CD_3OD and $CDCl_3$ solvents, respectively. Samples were then mixed well and filtered through 0.45 μm PTFE filters (13 mm, catalog number: 14232-754, VWR International LLC., Arlington Heights, IL, USA). Finally, filtered samples (0.5 mL) were added to clean NMR tubes (5 mm). ^{1}H NMR (128 scans) and ^{13}C NMR (8000 scans) were recorded. Proton ^{1}H and two-dimensional (2D) ^{1}H-^{1}H correlation spectroscopy (2D COSY) and ^{1}H-^{13}C heteronuclear single quantum coherence (2D HSQC) spectra (Appendix Figure B.3. to B.13.) were used to determine the prominent biomolecules in purified alfalfa smoke solutions having significant seed germinating bioactivity. Chemical shift (δ) values were reported in parts per million (ppm) in CD_3OD, $CDCl_3$ or D_2O. Signals are defined as singlet (s), doublet (d), triplet (t), and quartet (q). Semiquantitative analyses were performed using ^{1}H spectra, which were integrated within the range of 0−9 ppm. The total integral was calibrated to 100. The aromatic content was determined by integrating the region between the 6.10 and 9.00 ppm chemical shift (Appendix Figure B.14.).

4.2.8 GC-ESI-MS analysis

For this analysis, only the bioactive neutral fraction (Figure 4.2) was used. GC–MS analysis was conducted using an Agilent 7890A gas chromatography that is interfaced with a time-of-flight (TOF) Mass Spectrometer (JEOL Ltd. Japan) . The gas chromatography has an autosampler (Agilent Technologies, Inc.). The mass spectrometer was operated in the electron impact (EI, 70eV) mode.

Separation was achieved using a 30 m × 0.25 mm (inner diameter), 0.25 μm (film thickness), DB-5 ms, 350 °C Max. temperature (Agilent J &W Scientific) column with ultra-high purity (UHP) helium as the carrier gas (constant flow, 0.8 mL/min) using an injection volume of 1 μL (split ratio of 100:1). The initial oven temperature was set to 40 °C and held for 1 min before increasing at 5 °C /min to 200 °C, then 10 °C /min to 320 °C and held for 10 min (inlet temperature 280 °C; transfer line 320 °C). The ion source was set at 300 °C, and the spectrometer was set to record 25 spectra per

second. MS detection was performed using the positive ion mode. A mass range from 35 to 640 m/z was scanned. Total GC running time was 59 min.

Data acquisition and possible chemical nature/structural identification were made by comparing the mass spectra (in EI mode) of the EtOAc neutral mixture with an experimentally obtained mass spectra in the NIST 17 library (Data Version: NIST v17, Software Version: 2.3) (https://chemdata.nist.gov/dokuwiki/doku.php?id=start). Only compounds with high scores (> 600) were selected for manual inspection (Simon-Manso et al., 2013).

4.2.9 Data analysis

All statistical analyses were performed using R v. 3.6.3 (R Development Team, 2020) and all experiments followed a completely randomized design (CRD). Germination percentage was analyzed using a General Linear Mixed Model (GLM) procedure within the package 'lme4' (version 1.1-23). The main effects and interactions between smoke dilutions and smoke fractions (resin, liquid-liquid extraction, or C18) on seed germination of bioassay species were treated as independent variables. Replicates and runs were factored into the model as random effects. Means were separated using the function 'difflsmeans' with the significance level of $\alpha = 0.05$ or $\alpha = 0.001$. All analysis tables are available in Appendix B1 – B3.

4.3 Results

4.3.1 Efficacy of resin-separated active fractions in bioassay germination

Seed germination of bioassay species, *L. sativa,* responded significantly to smoke water resin-separated treatments ($P<0.001$), its dilutions ($P<0.001$) and the interactions of resin treatments × dilutions ($P<0.01$). All three fractions significantly increased lettuce seed germination in darkness ($P<0.05$) compared to distilled water control and/or ethanol control, respectively (Figure 4.3).

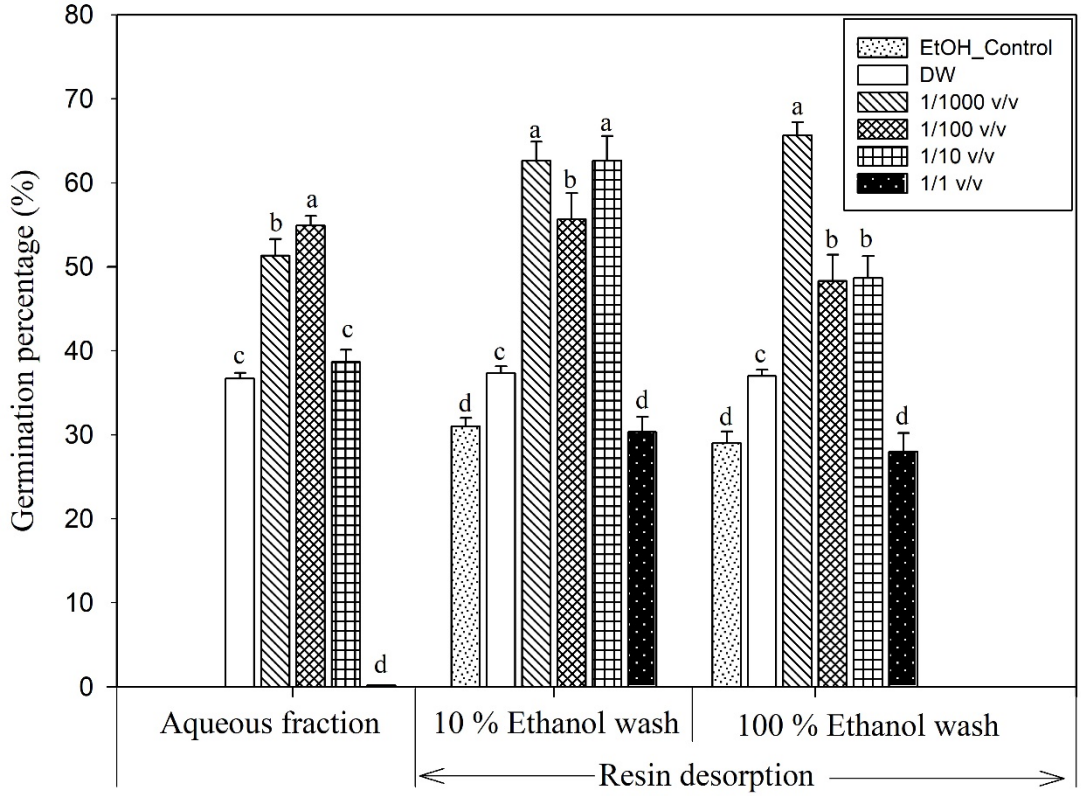


Figure 4.3 Germination percentages after lettuce seed bioassay. Seeds treated with distilled water or serial dilutions of alfalfa aqueous smoke water after FPX-66 resin separation and incubating at 25/15 °C in 24 h darkness for one day (EtOH: Ethanol, DW: HPLC grade distilled water as the control). Means with different lower-case letters within a resin fraction indicate germination of treated seeds were significantly different ($P \leq 0.05$) (means ± S.E., n = 10).

Bioactivity in an aqueous fraction explains the possibility of having active chemical compounds with more polar bonds compared to the other two desorption fractions (10% and 100% ethanol wash). Treating seeds in the 1/1000 and 100 v/v dilutions of aqueous fraction increased the germination percentage significantly by 29% and 33%, respectively, compared to the control (Figure 4.3). However, treating seeds in 1/10 v/v smoke dilutions did not increase germination percentage, while 1/1 v/v treatment reduced the germination percentage to 0% relative to control in 24 h darkness. This suggests toxic effects in higher aqueous concentrations on seed germination in darkness.

The 10% ethanol wash resulting from resin desorption gave a significant ($P<0.05$) increase in the germination of bioassay species, indicating that fewer non-polar chemical compound/s may have increased seed germination. Dilutions of 1/1000 and 100 as well as 1/10 v/v increased the germination percentage significantly compared to the distilled water (DW) control (by 40%, 33%, and 40%, respectively). Treating seeds in 1/1 v/v dilutions reduced the germination percentage by 19% relative to the DW control.

The 100% ethanol wash solution also increased the lettuce seed bioassay germination significantly, suggesting that more non-polar compounds are equally responsible for seed germination of this bioassay species. Treating seeds with 1/1000, 100, and 10 v/v dilutions of the aqueous fraction increased the germination percentage significantly (by 44%, 23%, and 24%, respectively), compared to the DW control. Treating seeds with 1/1 v/v dilutions reduced the germination percentage by 24% relative to the DW control.

Although inconclusive, Figure 4.4 (b) shows a proton signals pattern similar to the standard Karrikin (KAR1) (Figure 4.3 a), which is a potent seed germination promotive compound isolated from smoke water (Flematti et al., 2004). Standard KAR1 shows the characteristic ^{1}H NMR: δ 1.87 (3H, s), 6.78 (1H, d), 7.57 (1H, d), 7.79 (1H, s) and my 100% aqueous fraction obtained after 2 hours of resin adsorption produced similar ^{1}H NMR (H_2O, 600 MHz) signals at δ 2.00 (3H, s), 6.58 (1H, d), 6.99 (1H, d), and 7.60 (1H, s).

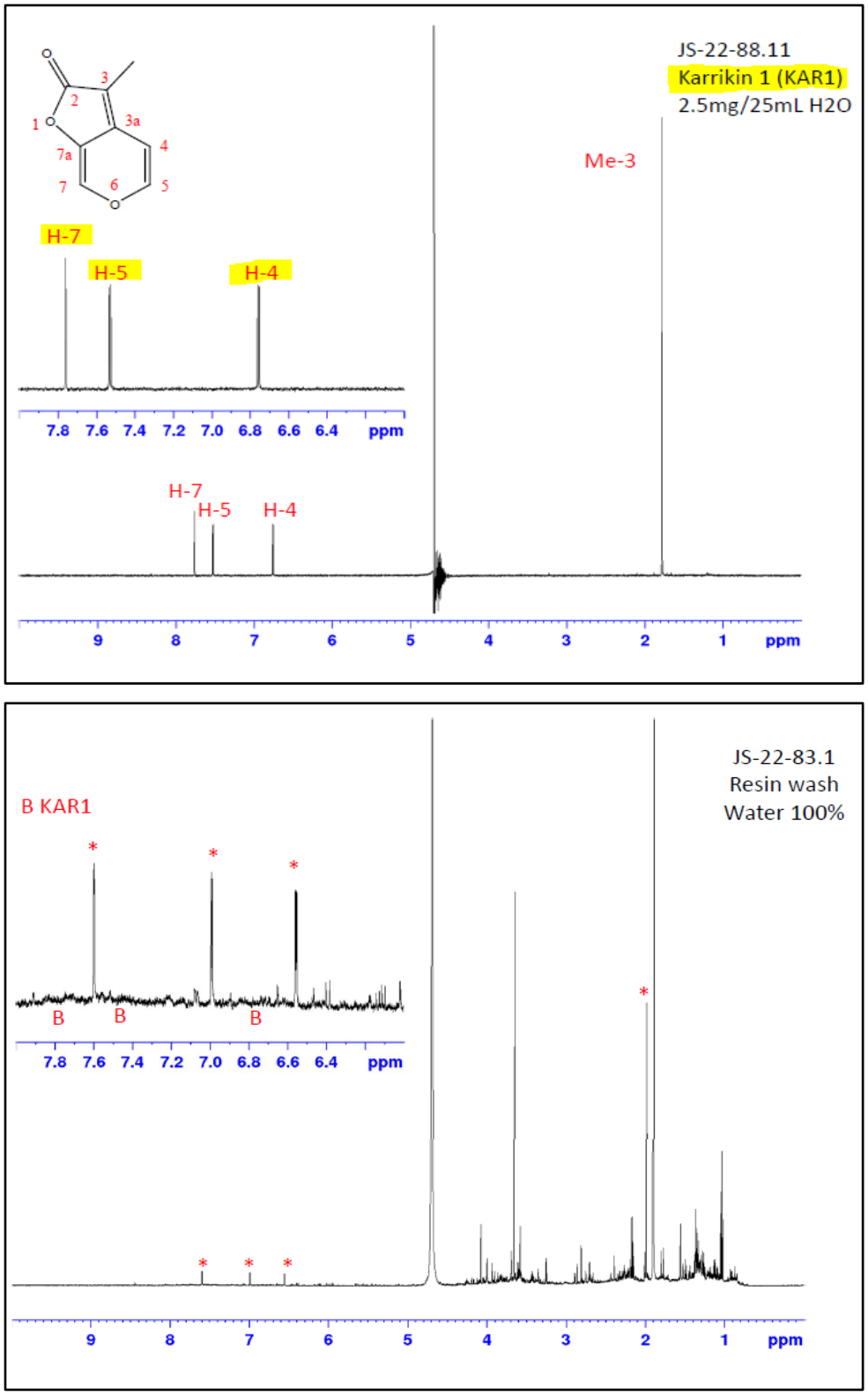


Figure 4.4 Comparative 1D ^{1}H NMR spectra obtained from (a) standard Karrikin in D_2O solvent and (b) resin-fractionated alfalfa 100% aqueous smoke water fraction in D_2O solvent.

4.3.2 Efficacy of liquid-liquid extracted fractions for bioassay germination

In a liquid-liquid extraction of smoke water, EtOAc separated the smoke solution made from alfalfa into a water (aqueous) fraction and EtOAc (organic) fraction (Figure 4.5). The aqueous fraction was not bioactive, while EtOAc fraction showed bioactivity—promoting seed germination. A series of dilutions of these two fractions was prepared and used in the Salad Bowl lettuce bioassay to determine the concentration effect on seed germination. EtOAc fraction dilutions, 1/1000 & 1/100 v/v significantly increased seed germination ($P<0.001$) by 36% and 39%, respectively as compared with the DW control. To check the EtOAc solvent effect on seed germination, only pure EtOAc solvent was used to treat seed germination. A series of dilutions of EtOAc were tested to see its effect on seed germination in its diluted conditions (data not shown) and EtOAc treated seeds were not germinated. Also, in all dilutions of EtOAc, seed germination was less than that of distilled water control, indicating that only bioactive chemicals promote seed germination, not the EtOAc solvent from the chemical separation process.

Separated aqueous fraction dilutions of 1/1000, 1/100, 1/10, and 1/1 v/v decreased the seed germination of Salad Bowl lettuce significantly ($P<0.05$) by 16%, 45%, 100%, and 100%, respectively, compared with the distilled water (DW) control. This indicated that the active compounds are possibly in the organic EtOAc phase not in the inorganic aqueous phase (Figure 4.5). Concentrations of all partitioned fractions were equivalent to that of 1/1 v/v alfalfa stock smoke solution.

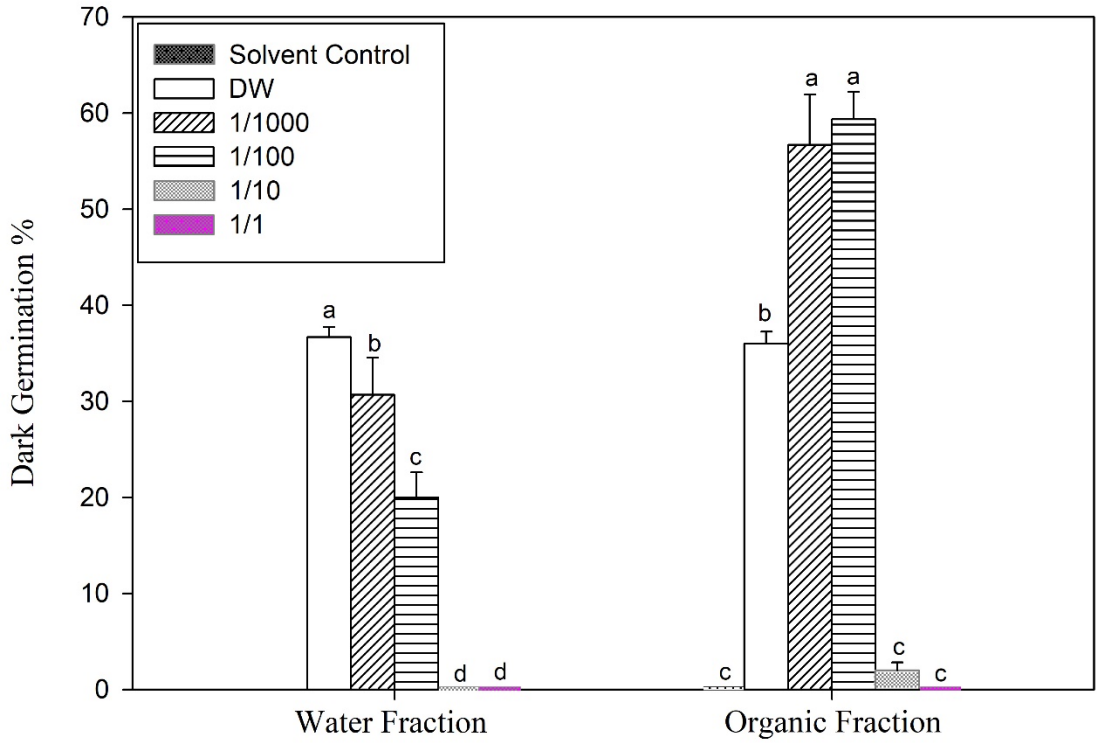


Figure 4.5 Germination of Salad Bowl lettuce (*L. sativa*) seeds after treating with water or organic (ethyl acetate) fraction of alfalfa aqueous smoke solution and incubating at 25/15 °C in 24 h darkness for one day. Solvent control; EtOAc in organic fraction, DW: HPLC grade distilled water as the control. Means with different letters indicate germination of treated seeds were significantly different ($P \leq 0.05$) within fractions (means ± S.E., n = 10).

The seed germination promotive EtOAc organic fraction was further divided into acidic and neutral fractions using the base, NaOH (Figure 4.6). Acidic fractions did not increase seed germination percentage, while neutral fractions significantly increased seed germination at alternate 25/15 °C compared with the distilled water (DW) control ($P<0.001$). Acidic fraction dilution of 1/1000 v/v did not change germination percentage, while 1/100 v/v dilution reduced seed germination percentage by 55% compared to the DW control. Neutral fraction treated seeds at both 1/1000 and 1/100 v/v dilutions increased the seed germination percentage significantly ($P<0.05$) by 40% and 31%, respectively.

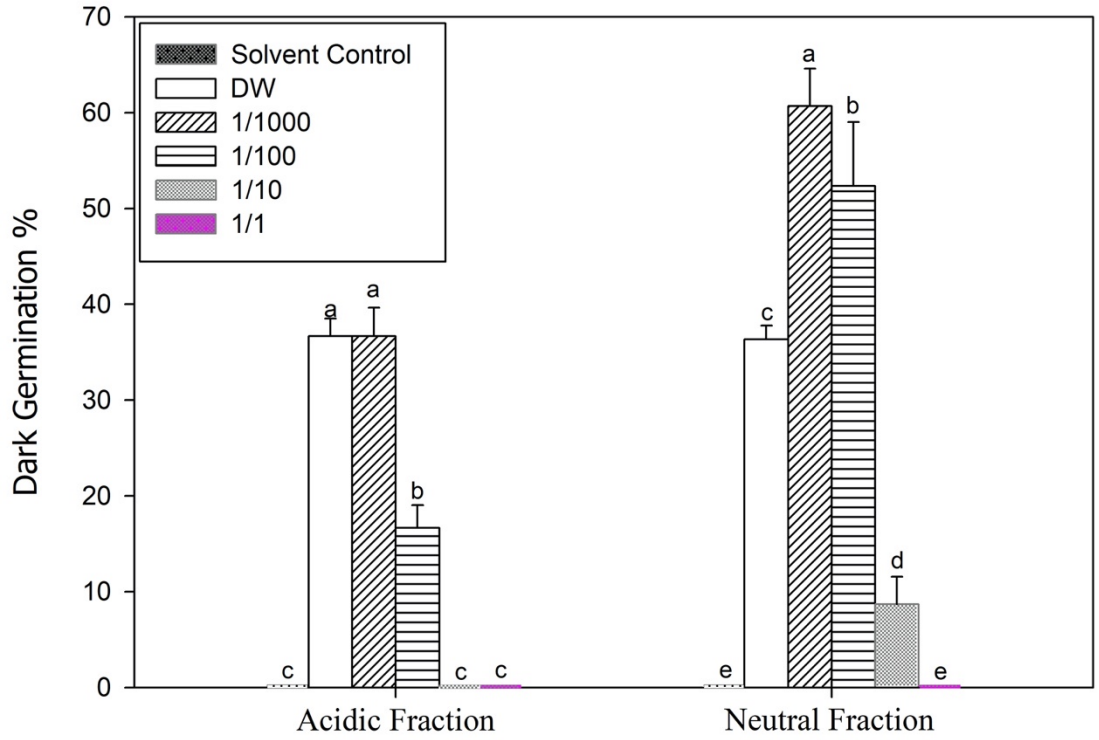


Figure 4.6 Germination of Salad Bowl lettuce (*L. sativa*) seeds after treating with an acidic or neutral fraction of alfalfa aqueous smoke solution and incubating at 25/15 °C in 24 h darkness for 1 day. Solvent control; NaOH in both fractions, DW: HPLC grade distilled water as the control. Means with different letters indicate total germination of treated seeds was significantly different ($P \leq 0.05$) within fractions (means ± S.E., n = 10).

To eliminate the NaOH solvent effect on seed germination, a series of dilutions of NaOH were tested (data not shown). In all NaOH dilutions, seed germination was less than that of distilled water control suggesting the non-significant effect of NaOH solvent seed germination.

4.3.3 Study of C18 column chromatography aided fraction efficacy using bioassay germination

An optimized fractionation process for smoke water was suggested by Flematti et al. (2008), where half of the vacuum concentrated neutral fraction (~55 mg) was directly used for tC18-reverse phase column chromatography, eluted with a methanol: water gradient (methanol proportion: 0%, 10%, 15%, 20%, 25%, and 100%) to further fractionate active from the neutral fractions.

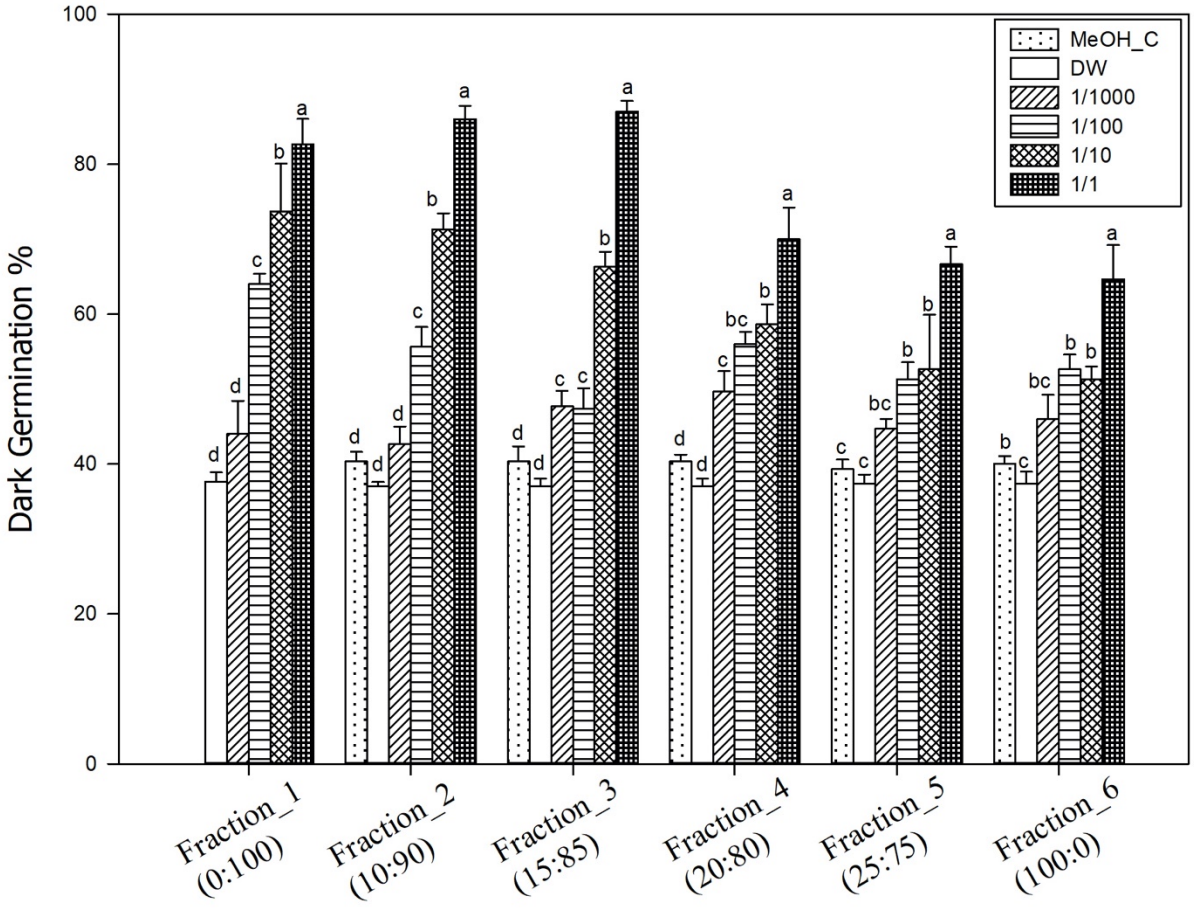


Figure 4.7 Germination of Salad Bowl lettuce (*L. sativa*) seeds after treating with water or serial dilutions of different fractions (methanol: water) derived from the tC18 - reverse phase chromatography of the neutral fraction from alfalfa or distilled water and incubated at 25/15 °C in 24 h darkness for one day. Means with different letters indicate total germination of treated seeds was significantly different (P≤0.05) within fractions (means ± S.E., n = 10).

All fractions were evaporated to dryness to remove the methanol solvent and then they were reconstituted with HPLC-grade distilled water. Each fraction was tested with a lettuce seed bioassay in a series of dilutions to assess the right concentration range for activity. All six fractions eluted showed a significantly increased lettuce seed germination in darkness ($P<0.05$) compared to distilled water control and/or methanol control (Figure 4.7), suggesting the presence of a variety of possible chemical compounds in the neutral fraction used in C18 column chromatography. In all eluted fractions, germination percentage was increased in ascending order from 1/1000 < 1/100 < 1/10 < 1/1 v/v dilutions, suggesting that different active chemicals or chemical mixtures are effective for seed germination in the presence of original eluted fraction concentration (1/1 v/v). Having 100% water in fraction 1 makes it the most polar fraction. As water percentage decreases, so does polarity in sequence from fraction 1 to 6. As a general trend, the germination percentage of seeds treated with all dilutions also decreased from fraction 1 to 6, indicating the presence of more polar active compound/s responsible for bioassay germination. Seeds treated with 1/1 v/v dilution from fraction 1 to 6, increased germination percentage significantly ($P<0.05$) by 54%, 57%, 57%, 47%, 44% and 42% compared to the distilled water control.

To eliminate the methanol solvent effect in each fraction on seed germination, a series of methanol dilutions were compared with lettuce seed bioassay (data not shown). In all methanol dilutions, seed germination was less than, or equal to, that of the distilled water control suggesting a non-significant effect of methanol solvent on seed germination.

4.3.4 HPLC analysis with KAR1 standard

The C18 fractions 1, 2, and 3 were separated using HPLC. Chromatograms of these three fractions were similar (only the 100:0 water: methanol fraction (fraction 1), EtOAc separated neutral alfalfa solution, and KAR1 (standard) are shown in Figure 4.8). Although the neutral smoke solution and C18 fractions show significant increase in seed germination, they did not show the peak at 2.436 min retention time, suggesting the absence of KAR1, a known germination promoting compound in alfalfa smoke water.

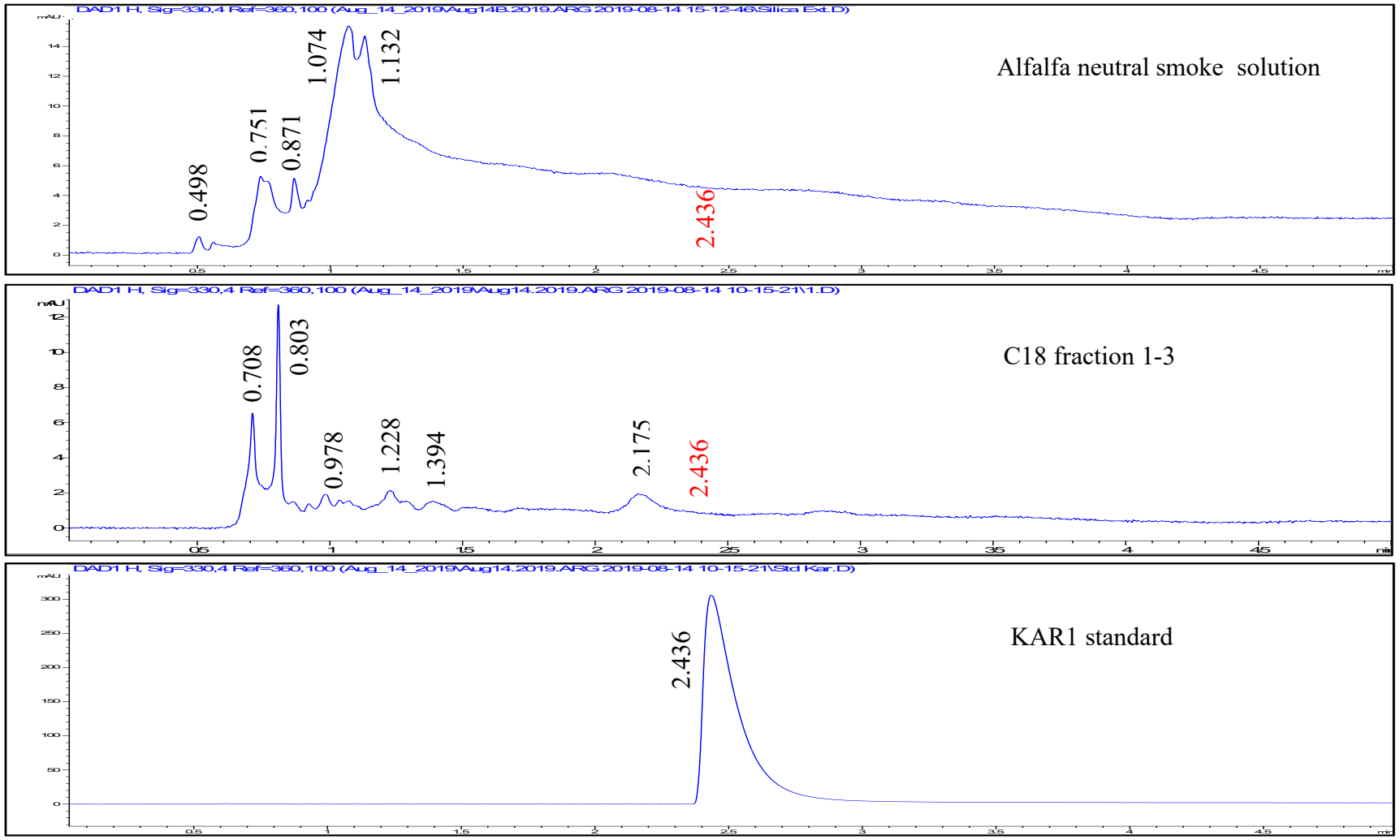


Figure 4.8 HPLC Chromatograms of the alfalfa neutral smoke solution (top), C18 separated; 100:0 water: methanol fraction 1 from alfalfa (middle) and KAR1 standard (bottom). Red color retention time (2.436 min) indicates the missing peak of KAR1 in those refined smoke solutions (top and middle).

Red-colored retention time (2.436 min) indicates the missing peak of KAR1 in the top and middle graphs compared to KAR1 standard (bottom chromatogram). Other peaks with retention times less than 2.436 min could be the chemical compounds responsible for promoting seed germination.

4.3.5 Comparison of bioassay germination responses to raw smoke water, isolated active fractions, KAR1 and GA_3

The lettuce seed germination bioassay responded differently to various alfalfa smoke fractions resulting from a range of separation techniques including resin purification, liquid-liquid layer separated neutral fraction, and tC_{18} reverse phase column chromatography (Figure 4.9). Also, known seed germination promotive chemical compounds, Karrikin (KAR1) and GA_3, were compared. In all seed treatments, lettuce seeds showed significant ($P<0.05$) seed germination except in 1/10 v/v EtOAc neutral smoke solution, 1/1000 v/v C_{18} purified smoke fraction, and GA_3 treatments, compared to the DW control at 25/15 °C in 24 h darkness incubation condition. Seeds treated with 1/1 v/v dilution of C_{18} purified alfalfa smoke gave the highest germination percentage (83%), which is a 55% increase in comparison with DW control. The resin separated fraction, raw smoke solution, EtOAc neutral fraction, and KAR1 increased the seed germination in similar percentages and significantly than DW control. Known seed germination stimulant, GA_3 treatment did show a promotive action on seed germination but a reduction of germination percentage by 35% as compared to DW control.

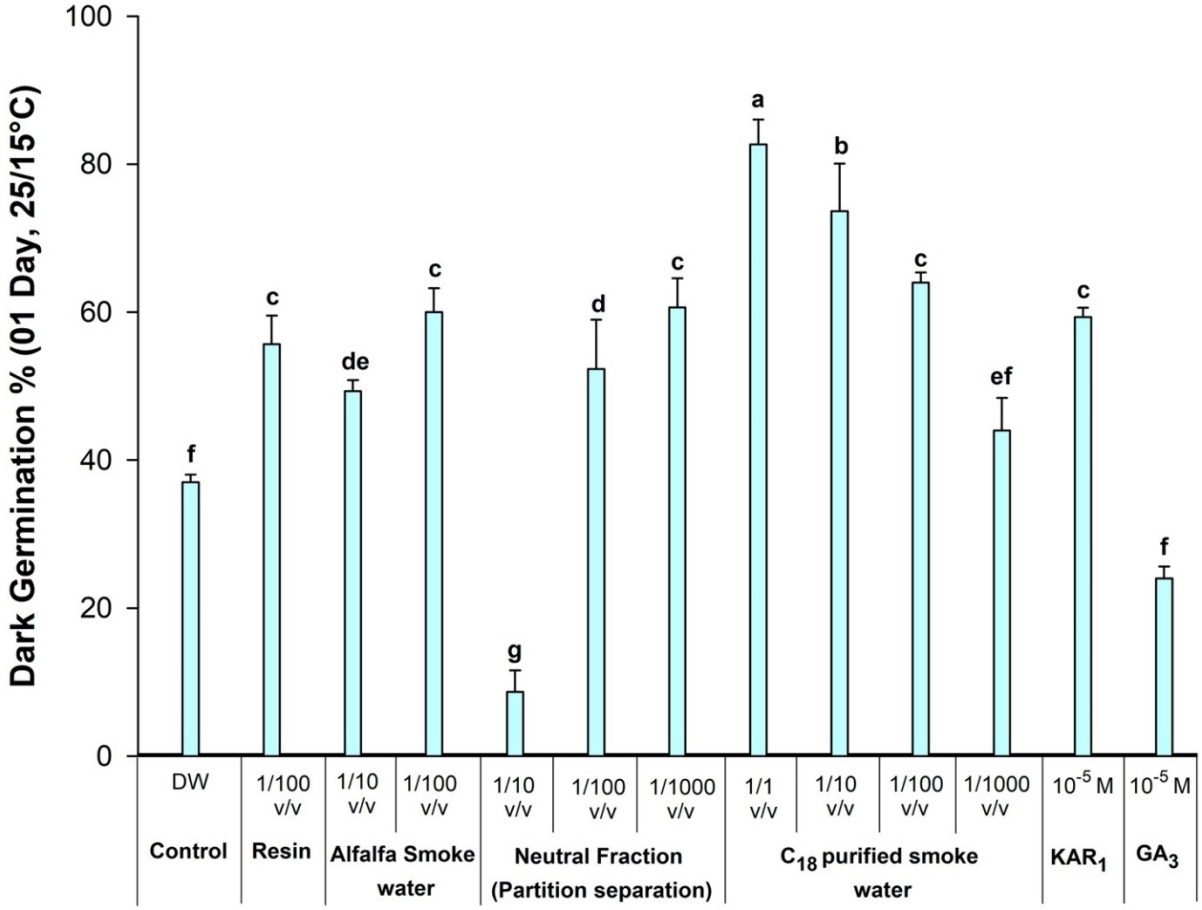
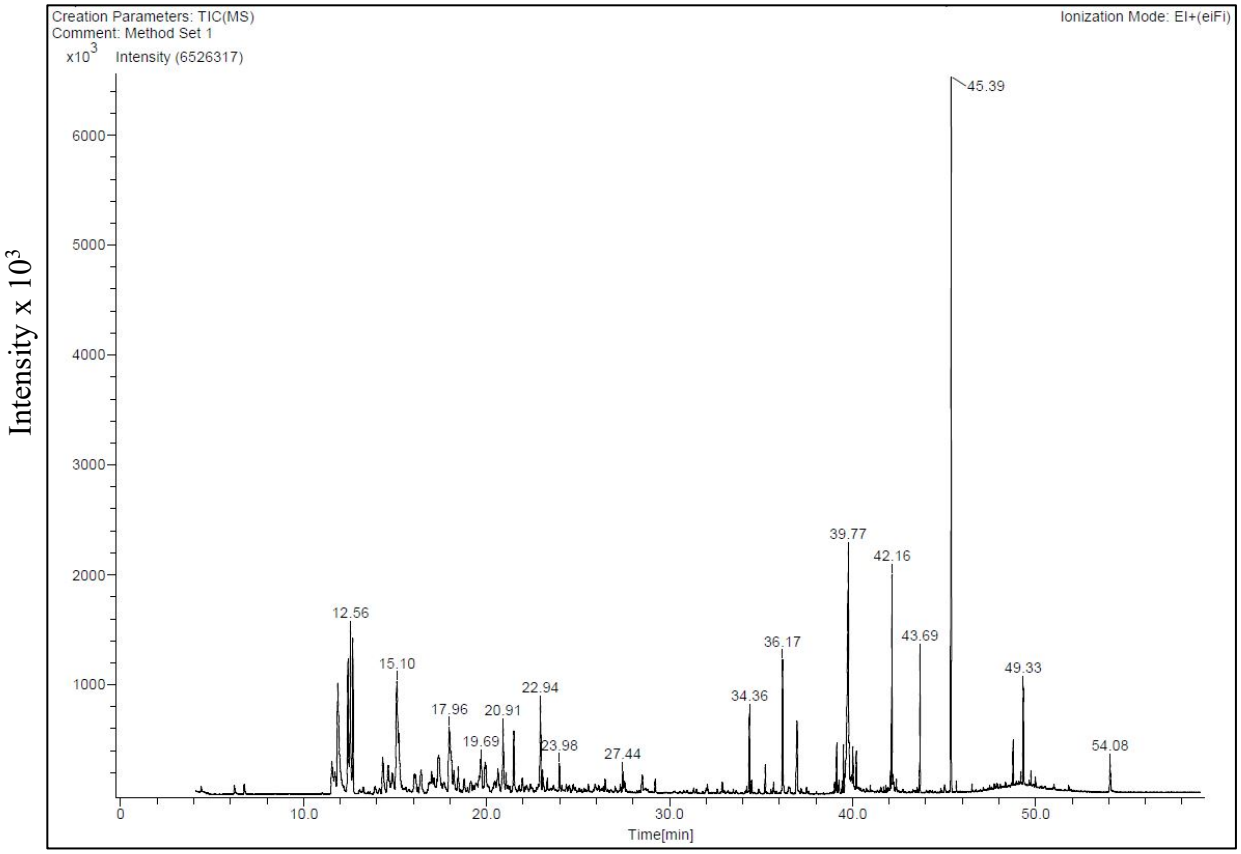


Figure 4.9 Comparisons of Salad Bowl lettuce (*L. sativa*) seeds germination response after treating with alfalfa smoke water, a resin-treated aqueous fraction, a partition separated smoke water neutral fraction, C_{18} purified smoke water (Fraction 01), KAR1, GA_3. DW: HPLC grade distilled water as the control and incubating at 25/15 °C in 24 h darkness for one day. Means with different letters indicate germination percentage of treated seeds were significantly different ($P \leq 0.05$) between seed treatments (means ± S.E., n = 10).

Since all C18 fractions were bioactive on the lettuce seed bioassay, further GC-MS library searches and NMR simulation studies (see section 4.3.6) were based on EtOAc extracted neutral alfalfa smoke fraction described in section 4.3.2.

4.3.6 Possible functional groups/chemicals identified in an isolated EtOAc fraction of alfalfa smoke solution

4.3.6.1 GC-MS analyses

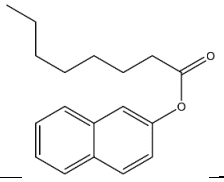
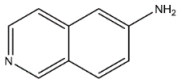
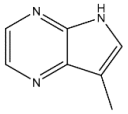
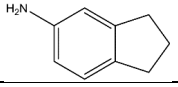
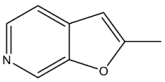
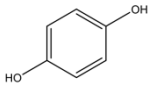
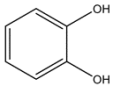
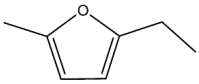
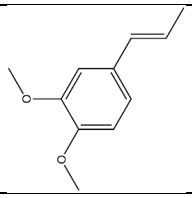
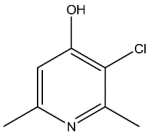


GC-MS retention time (min)

Figure 4.10 GC-MS Total ion chromatogram (TIC) of EtOAc extract of alfalfa smoke water (Karrikin locates closer to the retention time 29.20 minutes).

The chemical nature of seed germination promoting bioactive EtOAc neutral smoke solution (section 4.3.2, Figure 4.6) was investigated using GC-MS fragmentation patterns matched against the available NIST Mass library search. With higher matching score criteria (>600), 10 compounds were detected in the GC-MS of EtOAc neutral smoke solution, as shown in Table 4.2.

Table 4.2 Chemical compounds identified using GC-MS fragmentation patterns in conjugation with NIST Mass library search (matching score > 600) in EtOAc neutral extract of an alfalfa smoke solution.

Name	Molecular weight (g/mol)	Structure	Retention time (min)
naphthalen-2-yl octanoate	270.16		12.56
isoquinolin-6-amine	144.06		12.56
7-methyl-5H-pyrrolo[3,2-b] pyrazine	133.06		15.10
2,3-dihydro-1H-inden-5-amine	133.08		15.10
2-methylfuro[2,3-c] pyridine	133.05		15.10
1,4-benzenediol [Hydroquinone]	110.03		15.35
1,2-benzenediol [Catechol]	110.03		15.35
2-ethyl-5-methylfuran	110.07		36.17
(E)-1,2-dimethoxy-4-(prop-1-enyl) benzene	178.09		43.69
3-chloro-2,6-dimethylpyridin-4-ol	157.02		23.98

Major compounds were aromatics with five or/and six-carbon ring structures. Non-synthetic and chemically stable 34 compounds with higher ion intensity (> 400 × 10^6) were

identified from the GC-MS chromatogram (Figure 4.10) over the retention times of 11.55, 12.56, 15.1, 15.35, 22.94, 23.98, 32.06, 36.17, 43.69 and 45.39 minutes. Appendix Table B.4. shows the compounds identified with matching score > 400 in the NIST library search.

Known standard Karrikin (KAR1) gave the characteristic molecular fragmentation pattern at the retention time of 26.04 minutes (Figure 4.11) with the same GC-MS conditions. However, at this retention time (26.04 min), KAR1 chemical compounds were not found in the GC-MS library search for the neutral EtOAc extract studied.

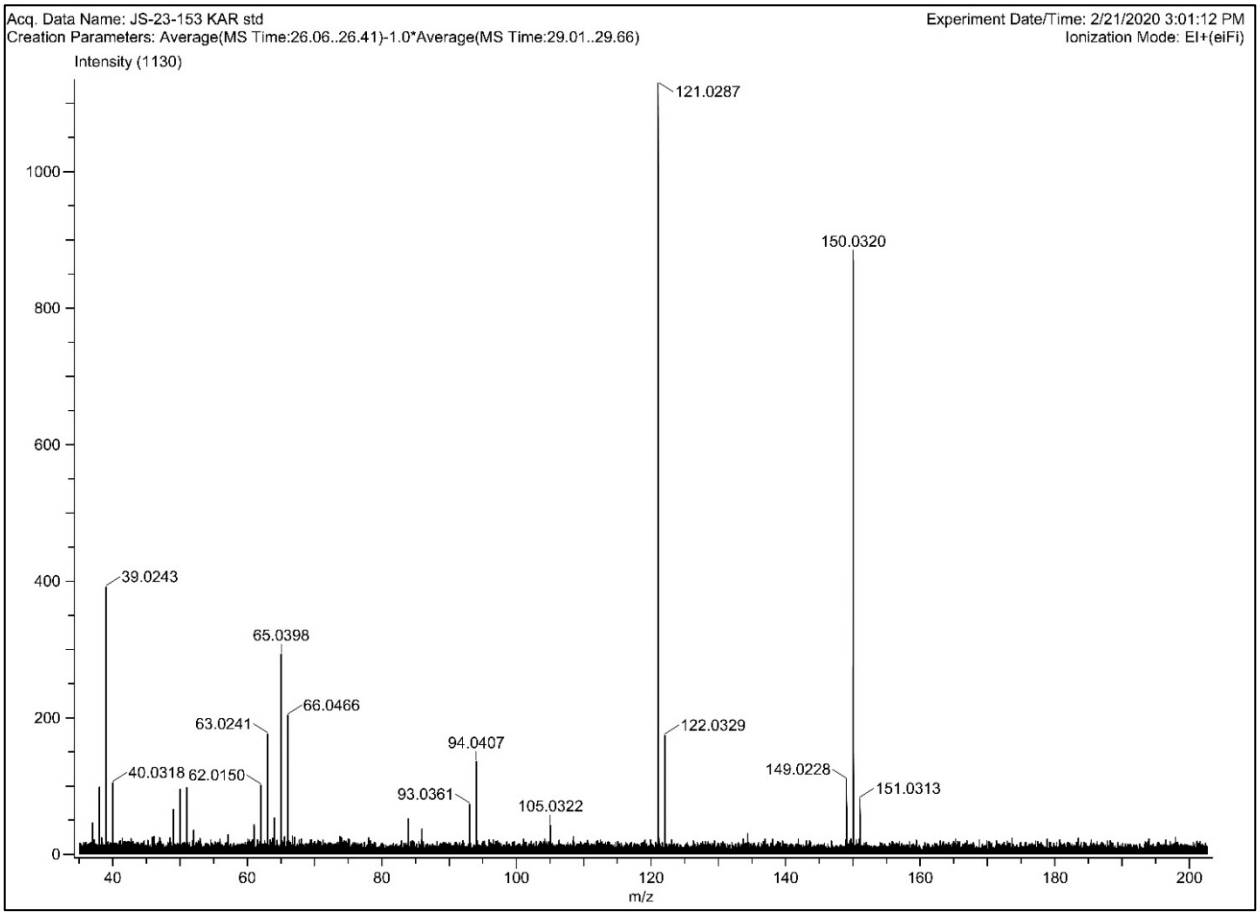


Figure 4.11 GC-MS chromatogram of standard Karrikin (KAR1) indicating the presence of m/z 150 and major fragment ion m/z 121 at 26.04 min retention time using the same GC conditions (GC-MS (ESI+): 150 (77),122 (17),121 (100), 66 (19), 65 (26)).

This is possible because not all chemical species are included in a single GC-MS library requires continuous updates. Alternatively, this KAR1 compound may be a minor component of our solution that remained undetected in this study.

4.3.6.2 NMR analysis

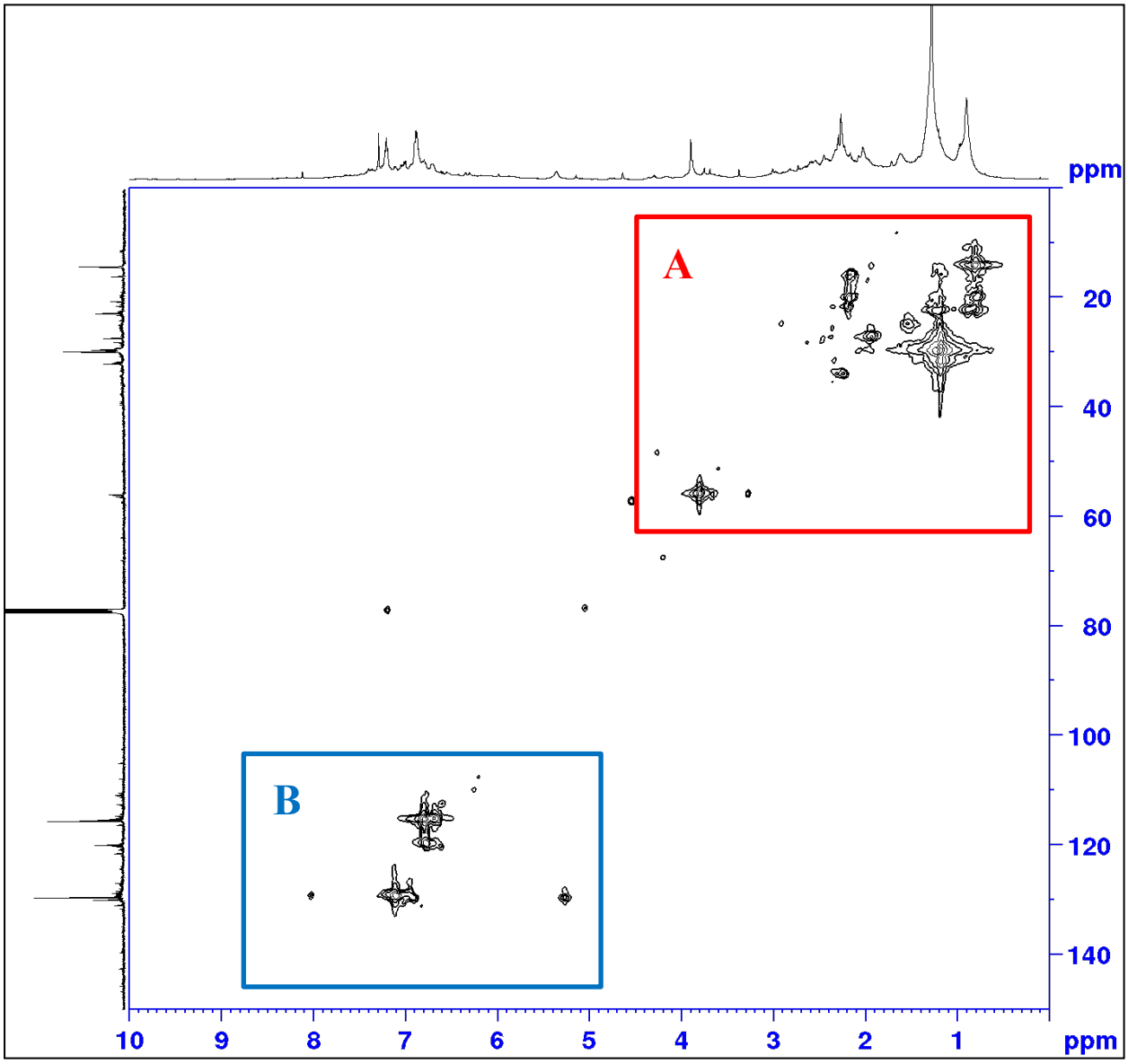


Figure 4.12 HSQC 2D NMR spectrum of the EtOAc extracted neutral fraction of alfalfa smoke solution (upper horizontal axis- ^{1}H NMR spectrum, left vertical axis- ^{13}C NMR spectrum) Region A and B represent major chemical compounds screened into two groups (see Table 4.3).

Table 4.3 Major chemical compounds identified using ^{1}H, ^{13}C, 2D COSY, 2D HSQC NMR spectra analysis of EtOAc neutral extract of alfalfa smoke and possible regions (A or B) of known smoke compounds located in Figure 4.12.

	Major chemical groups in HSQC NMR graph (Figure 4.12)	
	Region A	Region B
Possible major compounds	Acetic Acid Acetic Acid Methyl Ester Propionic Acid 1-Hydroxy-2-Butanone 3-Furanol, Tetrahydro	2,3,5-Trimethoxytoluene Benzoic Acid Phenol Phenol,2-Methoxy Phenol,3-Methoxy Phenol,2,6-Dimethoxy Phenol,3,4-dimethoxy Phenol,3,6-Dimethoxy 2-Methoxy-4-Methylphenol 2-Furancarboxaldehyde (Furfural) 1,4-Benzenediol (hydroquinone)** 1,2-Benzenediol (Catechol)**
Possible presence of previously identified and characterized compounds in smoke	3,4,5-trimethylfuran-2(5H)-one Glyceronitrile	Karrikinolide (KAR1) 1,4-Benzenediol (hydroquinone) 1,2-Benzenediol (Catechol)

** Both GC-MS and NMR analysis identified the possible presence of these active compounds

The HSQC NMR spectrum shows a correlation between ^{1}H and ^{13}C NMR spectra (Figure 4.12) that enables me to predict the presence of major chemical compounds in bioactive alfalfa smoke solution studied here.

Region A in the red box (Figure 4.12**)** largely consists of aliphatic chemical compounds with functional groups such as carboxylic acids, ketones, esters, etc. (Table 4.3). Region A (0.70 – 4.70 ^{1}H and 10 – 64 ^{13}C in ppm) includes signals mainly from CH_3 groups. Possibly part of these signals come from CH_2 groups in aliphatic compounds (C=O, C=N bonds, etc.). Especially within region A, in the direction of region B (3.0 – 4.7 ^{1}H and 50 – 64 ^{13}C in ppm), are predominantly signals from CH_2 groups in the vicinity of aromatic compounds and functional groups. More specifically, CH_2 groups of aromatic/phenyl rings may have a signature in region A in the direction of region B (Figure 4.12).

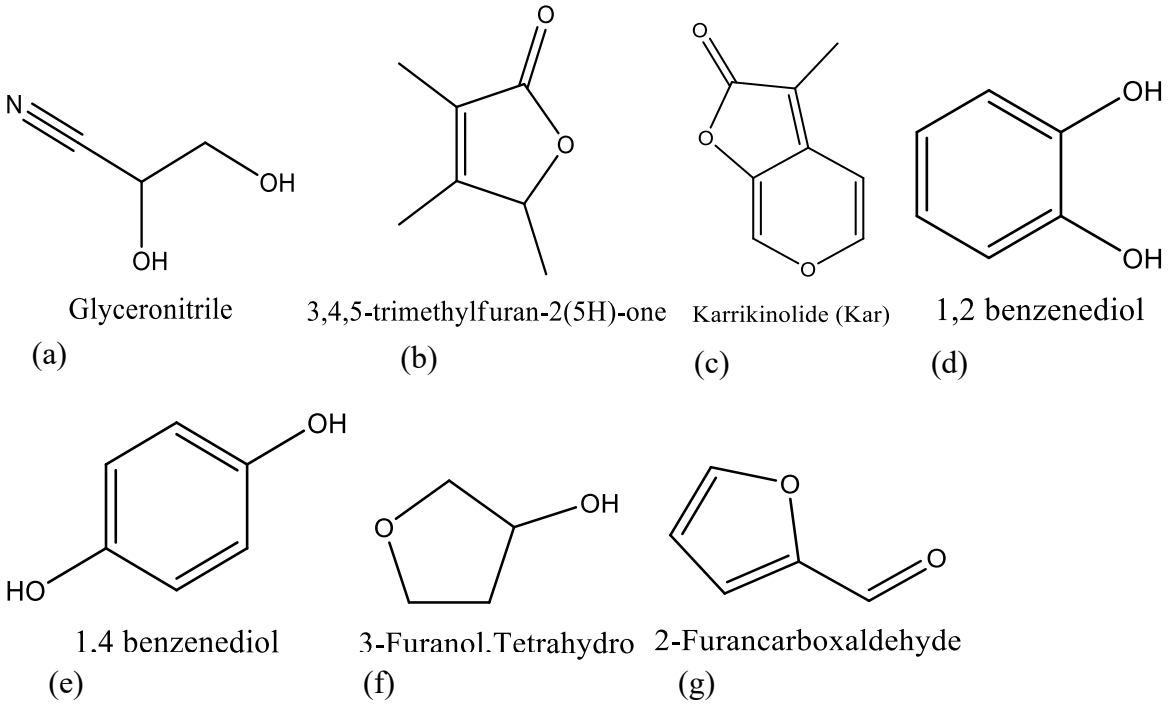


Figure 4.13 Chemical structures of known compounds in smoke water (a to e) and some of the possible compounds (d, e, f, g) identified through NMR analysis and simulation models.

A previously identified active compound, glyceronitrile (Flematti et al., 2011a), also belongs to region A due to its aliphatic chemical structure and NMR data (^{1}H NMR: δ 3.63 (2H), 4.54 (1H)). In addition, a four-carbon ring structure, 3-Furanol, Tetrahydro (^{1}H NMR: δ 1.90 (1H), 2.25 (1H), 3.60 (1H), 3.74 (1H), 3.88 (2H), 4.03 (1H)) was identified in region A, indicating the possible presence of a seed germination inhibiting compound, 3,4,5-

trimethylfuran-2(5H)-one (trimethylbutenolide; TMB) (^{1}H NMR: δ 1.49 (3H), 1.82 (3H), 2.42 (3H), 5.10 (1H)) with a four-carbon lactone, characterized by Pošta et al., (2017, 2018).

Blue color region B (5.20 – 8.10 ^{1}H and 107 – 138 ^{13}C in ppm; Figure 4.12) is characterized by peaks largely from aromatic CH and some from $=CH_2$ groups (Table 4.3, Region B). The main functional groups in this region are alcoholic -OH, -COOH and aldehyde (e.g., 2-Furancarboxaldehyde). Having many aromatic compounds with -CH groups in region B resulted in considerable overlap of peaks. Known seed germination promotive compounds (Figure 4.13), KAR1 (^{1}H NMR: δ 1.87 (3H), 6.88 (1H), 7.5 (1H), 7.80 (1H)), 1,4-Benzenediol (hydroquinone) (^{1}H NMR: δ 6.84 (3H)) and 1,2-Benzenediol (Catechol) (^{1}H NMR: δ 6.85 (2H), 7.24 (2H)) belongs to this aromatic compound region B. The 1,2-Benzenediol (Catechol) (Figure 4.15) and 1,4-Benzenediol (hydroquinone) (Figure 4.14) were identified as possible active compounds in alfalfa smoke water. These compounds were previously reported by Wang et al. (2017) and Kamran et al. (2017), respectively, as promotive chemical species for seed germination and/or seedling growth.

In semiquantitative analysis, the aromatic content was determined as ~50% in the EtOAc neutral smoke solution.

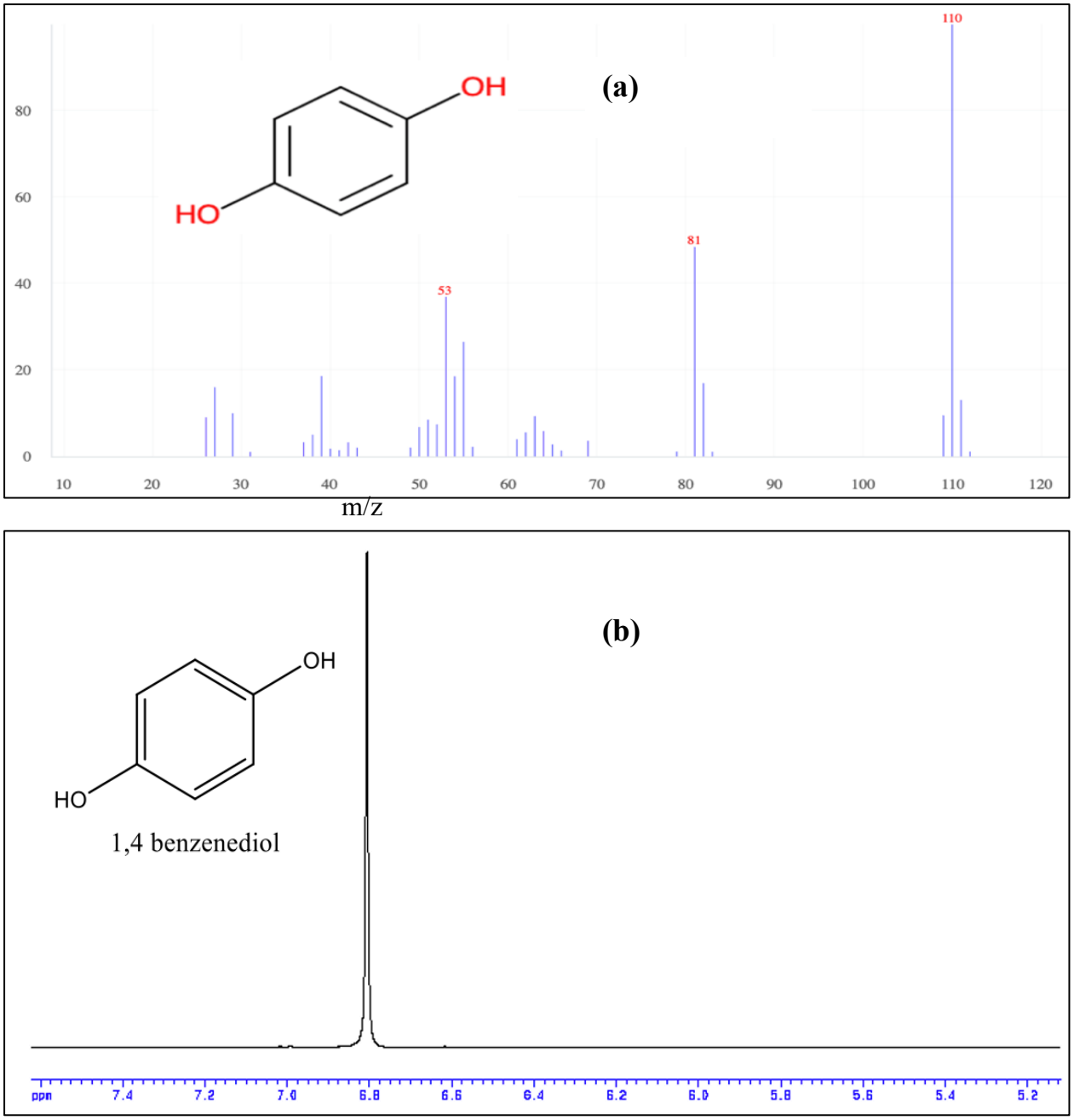


Figure 4.14 Possible active chemical compound, 1,4 benzenediol (Hydroquinone, 110.03 g/mol) screened from EtOAc neutral alfalfa smoke solution through both a GC-MS library search (a) mass chromatogram, GC-MS (ESI+): 53, 81, 110 and NMR simulation (b) ^{1}H NMR: δ 6.84 (3H).

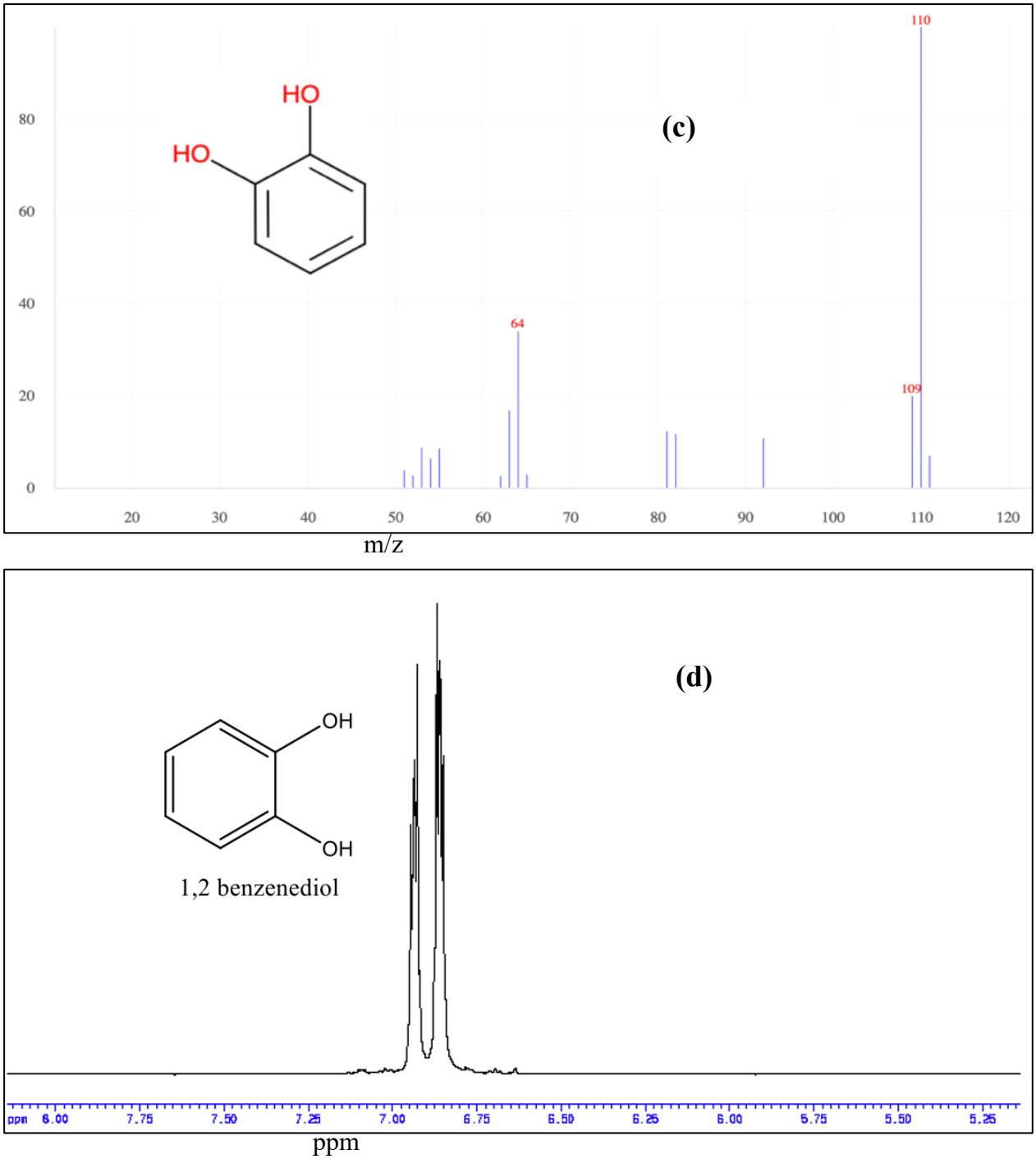


Figure 4.15 Possible active chemical compound, 1,2 benzenediol (Catechol, 110.03 g/mol) screened from EtOAc neutral alfalfa smoke solution through both GC-MS library search (c), mass chromatogram (GC-MS (ESI+): 64, 109, 110) and NMR simulation (d) ^{1}H NMR: δ 6.85 (2H), 7.24 (2H)).

4.4 Discussion

4.4.1 Resin separated the alfalfa smoke solution into active fractions that stimulated germination

The use of macroporous resin on active compound(s) extraction and isolation from plant-derived smoke has not been previously documented. In my study, synthetic resin FPX-66 was used as the adsorbent to explore the efficacy and utility of the fractionation process of alfalfa smoke solution into bioactive portions. This showed significant seed germination response using a lettuce seed bioassay. Non-polar resins have been successful in extracting valuable plant metabolites such as anticarcinogenic flavonoid hesperidin from Mexican limes and citrus (Scordino et al., 2003; Padilla et al., 2018), flavonoids from mulberry leaves (Wang et al., 2008), vitexin and isovitexin from pigeon pea (Fu et al., 2007), lycopene from tomato skins (Liu et al., 2010a) and polyphenols from apple juice (Saleh et al., 2008; Kammerer et al., 2007, 2010). I found that resin fraction screening was achieved using a lettuce seed bioassay because the available promotive compounds can substitute the light requirement of photoblastic *L. sativa* seeds incubated in the dark growth chambers (Drewes et al., 1995; Flematti et al., 2004; van Staden et al., 2004).

All three fractions significantly increased lettuce seed germination in darkness (Figure 4.3) compared to the distilled water or ethanol control, suggesting the possible presence of different active compounds in these three resin fractions with contrasting polarity. In addition, three unique NMR proton signals from the three resin fractions (Figure 4.4 b, Appendix Figure B.1. and B.2.) emphasizes that different chemical compounds are present in each fraction and exclude the possibility that any two given fractions contain the same compounds.

More non-polar (hydrophobic) chemical compounds in alfalfa smoke water were adsorbed into the resin, leaving the aqueous fraction with more polar (hydrophilic) compounds that do not interact with resin (Monsanto, 2015). In previous research, non-polar or hydrophobic compounds such as catechins (polyphenolic) (Vuong et al., 2010), anthocyanin (Kammerer et al., 2005), theaflavins (Monsanto, 2015) were adsorbed into non-polar polystyrene divinylbenzene (DVB-PS) based resins to which FPX-66 belongs (Soylak et al., 2001). Polarity, hydrophobicity and bond-forming (hydrogen bonding) are the main factors driving the adsorption process of

chemicals into a resin. Ethanol (EtOH) at different concentrations is the most often recommended solvent in the desorption step of non-polar resins such as FPX-66, where the release of adsorbed chemicals stepwise is achieved sequentially (Monsanto, 2015). In this solvent swing system, the adsorption equilibrium in sorbates is shifted by replacing the water-based solvent (adsorption) with ethanol-based (desorption). Water molecules are more polar in nature compared to pure EtOH solutions. Therefore, a 10:90 EtOH: water solution is more polar than a 100:0 EtOH: water solution. Since fewer non-polar or hydrophobic molecules are released first from the non-polar adsorbent FPX-66, the more polar 10% EtOH solution was used first in the desorption/washing process. The resulting resins may contain non-polar molecules, which are eluted from the resin with non-polar solvents. Finally, after the second desorption, a 100% EtOH wash will elute most of the non-polar molecules that were adsorbed in resin. However, in my study, adsorption capacity and adsorption/desorption rates were not calculated as I did not focus on a single chemical species. Typically, resin adsorption studies investigate a known chemical compound/s or a broad group of chemicals.

Regardless of the resin fraction type (i.e., aqueous, 10% EtOH, 100% EtOH), the lettuce seed bioassay showed a significant germination response in dilutions of 1/1000 and 1/100 v/v. This indicates the possibility of different chemical compounds with different polarities in alfalfa smoke water. Ren et al. (2017) reported the presence of different bioactive chemical compounds that stimulate seed germination in alfalfa smoke water but at different steps of chemical separation.

However, all original resin fractions (1/1 v/v) showed a toxic (aqueous fraction) or non-significant (10% EtOH, 100% EtOH fractions) effect on bioassay germination percentage. This could be due to the presence of inhibiting or toxic compounds in smoke at relatively high concentrations (Dixon & Roche, 1995; Kulkarni et al., 2007) or different chemicals exhibiting activity at different concentrations (Boucher & Meets, 2004). The germination response of bioassay seeds increases after diluting the original fractions. This finding agrees with studies that have shown that applying diluted concentrations of smoke water improves seed germination in several species (Light, 2006). Studies of photoblastic *Apium graveolens* seeds found germination to be induced with diluted smoke solutions (Thomas & van Staden, 1995). It could be possible that dilution may eliminate the possible toxic or inhibitory effect of chemicals present in smoke

water and promote seed germination (Brown & van Staden, 1997; Preston & Baldwin, 1999; Light, 2006). In natural environments, if inhibiting compounds are present, their effect may be neutralized through dilution or leaching by rainwater (Baldwin et al., 1994).

FPX-66 is a non-ionic, non-functional, aromatic polymer (Yangui et al., 2017), which is effective at adsorbing non-polar molecules such as phenolics (Monsanto, 2015). The 10% and 100% EtOH fractions may contain phenolic compounds, which induce increased seed germination. NMR and GC-MS data also provide evidence (see section 4.3.6) for the presence of phenolic compounds such as hydroquinone (Kamran et al., 2017) and catechol (Wang et al., 2017) (Figures 4.14, 4.15).

Karrikin (KAR1) is a potent seed germination stimulant and the active compound in smoke solution. Former studies demonstrated that KAR1 can be isolated through a continuous bioassay-guided fractionation process and confirmed by chemical synthesis (Flematti et al., 2004, 2015). KAR1 contains a butanolide group that is a specific type of lactone fused with a pyran ring with characteristic ^{1}H NMR signals (Flematti et al., 2015). Interestingly, a polar or hydrophilic 100% aqueous resin fraction of alfalfa smoke solution exhibited similar ^{1}H NMR signals in singlet(s) and doublet(d), giving a possible clue for the presence of this active compound. Minor differences in the chemical shifts were observed in comparison to standard KAR1 molecules. These minor chemical shifts could be possible because magnetic energy absorption by the nucleus is a characteristic of the surrounding chemical environment (Colegate & Molyneux, 2007). The chemical environment in alfalfa smoke water solution is completely different from that of standard KAR1 solution. In contrast, this KAR1-like NMR signal could be due to other types of chemical compounds; I did not confirm the presence of KAR1 by conducting further testing of this 100% aqueous fraction using other supportive techniques such as mass spectrometry (MS) or HPLC. Therefore, these preliminary results provide the foundation for further research on resin separation of active compounds from smoke solutions.

Previous studies have found that KAR1 can promote seed germination of light-sensitive seeds, including *L. sativa* (Flematti et al., 2004; van Staden et al., 2004), *Angianthus tomentosus*, *Gnephosis tenuissima*, *Myriocephalus guerinae*, *Podolepis canescens*, and *Rhodanthe citrina* (Merritt et al., 2006) under suboptimal light or darkness conditions. In my study, KAR1 enhanced

germination of Salad Bowl lettuce seeds, while GA_3 had no effect on seed germination percentage.

Mechanisms of KAR1 induced seed germination were suggested through two possible pathways. The first is under the control of seeds' phytochrome responses where active compounds in smoke solutions substitute for the light requirement in germination (Drewes et al., 1995; Light, 2006). There is evidence that active compounds in smoke solution may convert the chromoproteins (P_r and P_{fr}) on the phytochrome system of some seeds in both directions. The conversion of P_r into P_{fr} in the dark by smoke solution increased the seed germination of light-sensitive Grand Rapids lettuce seeds (Light, 2006). In contrast, light insensitive *Syncarpha vestita* seeds germinated in the light after a smoke solution treatment (Brown, 1993a).

The second mode of action of KAR1 is due to plant hormone effects on seed germination. KAR1 may stimulate germination in primed seeds by behaving like known plant hormones such as cytokinin and auxin (Jain et al., 2008) or by activating the seed's hormone system (Kulkarni et al., 2007). Two positively photoblastic species, *L. sativa* and *Nicotiana attenuate* exhibited an increased level of GAs and decreased ABA levels in seeds after smoke solution application (Gardner et al., 2001; Schwachtje & Baldwin, 2004). Also, it has been reported that KAR1 may increase the responsiveness of GAs and decrease the perception of ABA inside the seed hormone network (Nelson et al., 2009).

4.4.2 KAR1is not a major component of alfalfa smoke solution but other bioactive phenolic compounds with seed germination stimulative activity may be present

To identify the possible active compound/s in alfalfa liquid smoke responsible for increased seed germination, I performed a classical liquid-liquid partitioning as the first step using EtOAc solvent. This partitioning is largely based on the different functional groups of chemical compounds in a smoke solution. In brief, an initial smoke solution is partitioned into an aqueous fraction, organic fraction, acidic fraction (NaOH soluble), and a neutral fraction (Flematti et al., 2008). If active chemicals are present, they may go to one or several of these fractions based on the polarity of the compounds in the smoke solution. The total EtOAc organic extract and subsequent neutral fraction showed the majority of observed bioactivity with lettuce seed but only at lower concentrations and at seed incubation conditions of alternating 25/15 °C in

complete darkness (Figures 4.5 & 4.6). This suggests that the active compounds in smoke solution could be neutral. However, previous research conducted using a neutral fraction of alfalfa smoke solution did not show positive lettuce seed bioassay results at constant 25 °C in darkness (Ren et al., 2017). It is possible that the smoke-treated lettuce seed may interact synergistically at an alternating but not a constant temperature. Another possibility is that active chemical compounds may stimulate seed germination only during alternate temperature conditions. Ellern & Tadmor (1966) reported that seed gemination stimulation is not always governed by alternating temperature. The use of 25/15 °C alternating temperature and lettuce seed germination stimulation in this study is consistent with Abu et al.'s (2016) recommendation for 25/15 °C alternating temperature as the optimum temperature for smoke-elicited lettuce seed germination.

I tested a series of different concentrations of EtOAc solvent in the bioactive neutral fraction with Salad Bowl lettuce seed bioassay to investigate the solvent effect on seed germination. However, none of the solvent concentrations increased lettuce seed germination. This suggests that the solvent used in extracting process has no impact as a seed germination stimulant.

In my study, six C18 fractions gave a significant lettuce seed germination response but to varying degrees and raised the possibility of the presence of different chemical compounds in the alfalfa smoke solution. In contrast, it is reasonable to think that the active compounds may be contaminated among those C18 six fractions. Previous research has also reported the presence of several C18 active fractions resulted from the neutral smoke solution (Flematti et al., 2008; Kamran et al., 2017). This explains the chemically complex nature of the neutral fraction of alfalfa smoke water (Flematti et al., 2008). Although all C18 fractions showed a significant lettuce seed bioassay, none of these fractions gave the KAR1 (standard) peak in the HPLC analysis (Figure 4.8), suggesting that the neutral fraction of alfalfa smoke solution does not contain Karrikins. This observation is consistent with previous work conducted by Ren et al. (2017), where they reported that alfalfa smoke solution lacks this potent KAR1 seed germination stimulant after HPLC analysis. In addition to KAR1, other chemical compounds in alfalfa smoke solution may play a major role in smoke-elicited seed germination. Hydroquinone and catechol are two alternative chemical compounds identified in smoke solutions that enhance germination

or/and seedling growth in *L. sativa* (Kamran et al., 2017; Wang et al., 2017). Since all C18 fractions showed a significant seed germination, in my study, the bioactive neutral fraction was considered for further studies such as NMR and GC-MS data analysis to provide a broader insight on the chemical nature of bioactive alfalfa smoke solution.

According to GC-MS analysis and library search, predominant chemical compounds present in alfalfa neutral smoke solution were those with five or/and six-carbon ring structures (Table 4.2). These results are consistent with the class and structure of chemical compounds found by Flematti et al. (2007, 2011b), Pošta et al. (2017), Wang et al. (2017), and Kamran et al. (2017), who isolated and characterized aromatic seed germination promotive compounds from smoke water solutions. Interestingly, previously identified germination promoting compounds, 1,4-benzenediol [Hydroquinone] (Kamran et al., 2017) and 1,2-benzenediol [Catechol] (Wang et al., 2017) were detected at retention time 15.35 minutes (Table 4.2 and Figure 4.14, 4.15). Some chemical compounds with similar chemical structures to catechol but with different redox reactivities have also been detected in smoke solution (Baldwin et al., 1994). Wang et al. (2017) reported that only catechol-elicited lettuce seed radicle and root hair elongation are prominent among three isomers of dihydroxybenzene (1,2-dihydroxybenzene - catechol; 1,3-dihydroxybenzene - resorcinol; and 1,4-dihydroxybenzene - hydroquinone). It has been assumed that catechol is a prominent chemical compound in liquid smoke responsible for eliciting root morphology, and it has been identified as a highly abundant component in smoke (Baldwin et al., 1994; Montazeri et al., 2013). Catechol was detected in soil samples collected after several months after a natural wildfire, despite its susceptibility to direct photolysis (Wang et al., 2017).

Another form of dihydroxybenzene isomer detected in alfalfa smoke solution is hydroquinone which was reported as a seed germination enhancer in peanut (Elwakil, 2003), lettuce (Kamran et al., 2017), and as a wheat seedling growth agent (Li et al., 2009). Even though I detected several possible compounds, GC-MS chemical libraries do not contain all chemical compounds. Indeed, it is possible that most of the compounds in alfalfa smoke solution do not get analyzed in the GC due to the extreme volatile conditions of the compounds of interest. ^{1}H and two-dimensional (2D) NMR techniques (^{1}H, ^{13}C, 2D COSY, 2D HSQC NMR) were used as a supportive piece of information to extract molecular signals that overlap with the GC-MS results. The NMR simulation study detected several possible compounds, including phenolics, catechol

and hydroquinone, that were previously described and detected in GC-MS. Considering all analytical evidence, the active compound in alfalfa smoke solution could be an aromatic phenolic compound. The standard KAR1 pure compound was tested with the same GC-MS methods, and KAR1 molecules gave their unique mass losses at 26.04 min retention time (Figure 4.11). However, the alfalfa smoke neutral fraction did not show GC-MS signals for KAR1 molecules, suggesting the absence of this seed germinating stimulant in alfalfa smoke solution.

4.5 Conclusions

The research hypothesis that the KAR1 is not present as a major chemical compound in alfalfa smoke solution was accepted. However, KAR1 may or may not be present as a minor chemical component; this will have to be determined in future research. There is evidence of possible phenolic compounds such as hydroquinone and catechol in alfalfa smoke solution as active chemicals that enhance lettuce seed germination in darkness. Further research will be needed to isolate both minor and major active chemical compounds using not only lettuce seeds as a bioassay species, but also using other fire-ephemeral seed species that show higher positive sensitivity to alfalfa smoke. The chemical nature of alfalfa smoke solution studied in my work provides the background for future methodology development for separation and isolation of target chemical compounds.

CHAPTER 5. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH

5.1 General Discussion of Results

One of the objectives of this study was to compare and validate bioactive alfalfa smoke solution (Ren & Bai, 2016a) using smoke solutions produced from various plant materials. From this, the expectation was to identify the physiological mechanisms of smoke-stimulated seed germination responses. I was particularly interested in how leguminous (alfalfa) smoke solutions exert different germination responses on different fire-ephemeral species and evaluating how different chemical analyses and detection tools could help identify possible active compounds in alfalfa smoke solution.

Smoke solutions produced by burning a variety of plant materials stimulated germination in lettuce (*Lactuca sativa* L.) of varying degrees in my study. Previous studies have documented that dilute smoke solutions provide a positive germination cue, while concentrated smoke solutions can inhibit germination. This phenomenon was observed throughout my study, with the exception of *Sinapis arvensis* L. seeds. Dual and contrasting chemical signals, such as promoting and inhibiting compounds, must be present in the same crude smoke solution (Light et al., 2010). In my study, the presence of possible inhibitory (acidic fraction) and promoter (neutral fraction) compounds were evident during chemical fractionation (Chapter 4). It was found that the stimulatory effect of smoke is irreversible (Baldwin et al., 1994), but the reverse of the inhibitory effect of smoke is possible (Brown et al., 1993; Light et al., 2002).

Seed germination is not mediated by a single hormone but a ratio of two antagonistic hormones: GA and ABA (Seo et al., 2009). Light regulates the expression of GA and ABA biosynthesis-related genes through phytochrome-related protein in seeds, PIL5 (transcription factor), and thereby regulates the concentration of these two antagonistic hormones (Feng et al., 2008). Although the exact crosstalk between smoke or KAR1 and phytohormones is still unclear (Meng et al., 2017), active chemicals in smoke may increase the sensitivity of phytohormone receptors (specially GA) during dark germination (van Staden et al., 1995c). This agrees with my observation where smoke replaced the light requirement during germination in darkness. However, in this study, exogenous application of GA did not increase lettuce seed germination compared to smoke treatment in darkness, suggesting active chemicals in smoke are more

effective than exogenous GA in eliciting promotive germination (Chapter 04, Figure 4.9). Temperature regulates GA and ABA pools inside seeds by the control of their biosynthesis genes and eventually seed germination (Toh et al., 2008).

The germination percentage of lettuce was lower at 25/15 °C (37%) than that at a constant 25 °C (53%). Ren (2015) reported that alfalfa smoke solution did not promote lettuce germination at 25 °C in darkness. However, the same alfalfa smoke solution elicited positive germination responses on several South African fire-ephemerals and wild mustard species. Previous studies have shown that some Canadian Fescue Prairie species responded positively to concentrated alfalfa smoke solutions (Ren & Bai, 2016b). This indicates that plants from fire-prone habitats need less smoke to stimulate seed germination compared plants from areas that are not fire-prone; species in different ecosystems have distinct sensitivities to germination cues (Santana et al., 2013), and seeds of different plant species may interact with different chemicals in the same smoke solution (Flematti et al., 2011a). Fire is a natural disturbance in grasslands that can facilitate high germination and seedling establishment (Keeley et al., 2012). Habitat fragmentation, fire suppression, human settlement in wildlands, and livestock grazing are the main anthropogenic factors that suppress natural and frequent surface fires (Dellasala et al., 2004). Small, fragmented grassland patches can be managed with prescribed fire or smoke solution applications to enhance seedling recruitment from soil seed banks (Yates & Ladd, 2005). The South African species tested in this study were collected from undisturbed savanna community that is known for fire/smoke responsiveness (Table 3.1). This, smoke technology appears to be a useful and innovative tool for restoration and grassland regeneration (Ghebrehiwot, 2010).

Crude alfalfa smoke solution and/or chemically separated fractions when applied to Salad Bowl lettuce seed, South African and wild mustard seeds significantly promoted seed germination in my study. Importantly, I did not detect KAR1 in these solutions, suggesting the presence of a different kind of active compound/s in alfalfa smoke solution. I predicted that the presence of previously identified phenolic compounds in plant-derived smoke solutions (hydroquinone and catechol) may act as active stimulants in alfalfa smoke solution.

5.2 Conclusions

The stimulating effects of smoke solutions on lettuce seed germination varied between alternating 25/15 °C and constant 25 °C. Diluted solutions of all smoke types substituted for the light requirement of germinating seeds at alternating 25/15 °C, but this effect was not noticeable at a constant 25 °C, which may reflect plant adaptations to field conditions in temperate regions. Smoke solutions prepared from South African plant species were as effective as those from alfalfa and wheat in enhancing lettuce seed germination. Hence, contrary to my first hypothesis, different smoke types stimulate seed germination responses more or less similarly within a given germination condition. However, the second hypothesis, that the different germination response is defined by environmental conditions, is accepted. Plant species tested in this study inhabit South African fire-prone habitats, and all, except for *H. capensis*, responded positively to diluted alfalfa smoke solutions. This demonstrated the positive effects of plant-derived smoke for managing rangelands and restoring degraded grasslands where natural fire disturbances have been suppressed. As seen in this study, the germination of *Sinapis arvensis* L. was enhanced by both alfalfa and wheat straw smoke solutions in my study, despite KAR1 not being a prominent chemical in alfalfa smoke solutions. Seed germination of this species was positively related to the concentration of smoke solutions indicating the requirement for high concentration smoke solutions to break dormancy as well as a high tolerance of potentially inhibitory compounds. Thus, consistent with my hypothesis, fire-ephemeral species respond more positively to alfalfa and/or wheat smoke.

Smoke also shows promise as a weed management tool, for its ability to expose dormant plants to herbicide exposure. However, leaching through soil profile and an observed requirement for highly concentrated smoke solution to stimulate some weed species, creates practical considerations for field applications. Chemical fractionation results indicate that KAR1 was not present as a major germination stimulant in alfalfa smoke solution, supporting my hypothesis that non-KAR1 chemical compounds may promote seed germination. The positive response of *Sinapis arvensis* L. make it a suitable bioassay candidate for isolating promotive compounds in alfalfa smoke solutions in future research.

5.3 Future Research

This study provided baseline information for future studies on the comparative stimulation of alfalfa smoke solution, among other smoke solutions, on seed germination. As well, my research indicates which, and how, different fire-ephemeral seed species respond to a leguminous alfalfa smoke solution. Upon further examination, I predicted that the possible chemical nature of active compounds in alfalfa smoke promotes seed germination not only in lettuce seeds but also other ecologically and agriculturally important fire-ephemeral species. However, the physiological mechanism of smoke-stimulated seed germination remains unclear, and future studies will need to focus on understanding how high smoke concentrations (alfalfa in particular) regulates the endogenous and antagonistic GA and ABA ratio within seeds while stimulating seed germination. This will add a further dimension to the emerging picture of a smoke-stimulated germination mechanism. In place of lettuce seeds, my use of wild mustard as a bioassay system during the chemical separation process will help uncover the unique chemical entity in alfalfa smoke solution. Smoke fractionation using resin might have potential to efficiently isolate germination stimulants but will require further testing. As well, the field application of alfalfa/wheat smoke solution will provide a true potential for stimulating dormant weed seed banks, wild mustard seeds in particular, in an uncontrolled environment. Fragmented grassland and degraded fields can be tested with fire or smoke solutions in future regeneration studies.

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APPENDICES

A. Chapter 3 Supplementary Tables

A.1. ANOVA tables for the effects of different aqueous smoke solutions on germination percentage and the number of days to 50% total germination of the bioassay species, Salad bowl lettuce (*L. sativa* L.), in the laboratory.

Table A.1.1. ANOVA table for the effects of 08 different aqueous smoke solutions on seed germination of *L. sativa*.

Source of variation	numDF	denDF	F-value	P-value
Temp	1	1040	255.17	<0.001
Light	1	1040	2767.70	<0.001
Type	7	1040	93.43	<0.001
Dilution	4	1040	4067.78	<0.001
Temp: Light	1	1040	0.61	0.4362
Temp: type	7	1040	34.18	<0.001
Light: type	7	1040	26.96	<0.001
Temp: dilution	4	1040	76.24	<0.001
Light: dilution	4	1040	227.87	<0.001
Type: dilution	28	1040	52.42	<0.001
Temp: Light: Type	7	1040	19.08	<0.001
Temp: Light: dilution	4	1040	59.75	<0.001
Temp: type: dilution	28	1040	15.59	<0.001
Light: type: dilution	28	1040	5.92	<0.001
Temp: light: type: dilution	28	1040	6.58	<0.001

Table A.1.2. ANOVA table for the effects of aqueous alfalfa smoke solution on the number of days to 50% germination of *L. sativa*.

Source of variation	numDF	denDF	F-value	P-value
Temp	1	210	290.10	<0.001
Light	1	210	712.69	<0.001
Dilution	3	210	174.33	<0.001
Temp:Light	1	210	60.04	<0.001
Temp:Dilution	3	210	35.60	<0.001
Light:Dilution	3	210	38.79	<0.001
Temp:Light:Dilution	2	210	0.69	0.500

Table A.1.3. ANOVA table for the effects of aqueous wheat smoke solution on the number of days to 50% germination of *L. sativa.*

Source of variation	numDF	denDF	F-value	P-value
Temp	1	196	5.49	0.0201
Light	1	196	301.37	<0.001
Dilution	3	196	179.87	<0.001
Temp:Light	1	196	28.99	<0.001
Temp:Dilution	3	196	31.54	<0.001
Light:Dilution	3	196	27.90	<0.001
Temp:Light:Dilution	2	196	7.60	<0.001

Table A.1.4. ANOVA table for the effects of smoke types, smoke dilutions, light and temperature on lettuce seed bioassay germination percentage (%) and germination rate (T_{50}).

Source of variation	df	Germination (%)	df	Germination Rate (T_{50})
Main effects				
Smoke type (S)	7	***	1	ns
Dilution (D) #	4	***	3	***
Light (L)	1	***	1	***
Temperature (T) ϕ	1	***	1	***
Interactions				
T × L	1	ns	1	***
T × S	7	***	1	***
L × S	7	***	1	***
T × D	4	***	3	***
L × D	4	***	3	***
D × S	28	***	3	***
T × L × S	7	***	1	ns
T × L × D	4	***	2	***
T × S × D	28	***	3	***
L × S × D	28	***	3	ns
T × L × S × D	28	***	2	ns

*, **, *** Significant at 0.05, 0.01 and 0.001 alpha probability, respectively.

ns=non-significant.

04 volume by volume (v/v) serial dilution from initial stock smoke solutions, HPLC-DW as the control

ϕ Alternating and constant temperature

A.2. ANOVA tables for the effects of aqueous alfalfa and/or wheat smoke solutions on total germination percentage of the seven species studied in the laboratory.

Table A.2.1. ANOVA table for the effects of aqueous smoke solutions on total germination of *E, curvula*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	81	75.59	<0.001
Light	1	81	1.85	0.178
Priming:Light	4	81	0.20	0.938

Table A.2.2. ANOVA table for the effects of aqueous smoke solutions on total germination of *I. hilaris*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	40	214.84	<0.001
Light	1	40	17.79	<0.001
Priming:Light	4	40	95.68	<0.001

Table A.2.3. ANOVA table for the effects of aqueous smoke solutions on total germination of *H. nudifolium*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	40	1217.71	<0.001
Light	1	40	64.38	<0.001
Priming:Light	4	40	283.43	<0.001

Table A.2.4. ANOVA table for the effects of aqueous smoke solutions on total germination of *C. pospischilii*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	40	1041.28	<0.001
Light	1	40	99.59	<0.001
Priming:Light	4	40	146.66	<0.001

Table A.2.5. ANOVA table for the effects of aqueous smoke solutions on total germination of *M. nerviglumis*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	40	7.56	<0.001
Light	1	40	29.54	<0.001
Priming:Light	4	40	16.05	<0.001

Table A.2.6. ANOVA table for the effects of aqueous smoke solutions on total germination of *H. capensis*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	36	1645.04	<0.001
Light	1	36	1921.07	<0.001
Priming:Light	4	36	386.50	<0.001

Table A.2.7. ANOVA table for the effects of aqueous smoke solutions on total germination of *S. arvensis*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	129	86.2866	<0.001
Light	1	36.62262	5.2075	0.0284
Type	1	129	34.1371	<0.001
Priming:Light	4	129	3.9785	0.0045
Priming:Type	4	129	6.9946	<0.001
Light:Type	1	36.62262	0.3482	0.5588
Priming:Light:Type	4	129	0.2539	0.9068

A.3. ANOVA tables for the effects of aqueous alfalfa and/or wheat smoke solutions on the number of days to 10% or 25% or 50% total germination of the seven species studied in the laboratory.

Table A.3.1. ANOVA table for the effects of aqueous smoke solutions on the number of days to 25% germination of *E. curvula*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	81	354.94	<0.001
Light	1	81	0.45	0.5060
Priming:Light	4	81	9.54	<0.001

Table A.3.2. ANOVA table for the effects of aqueous smoke solutions on the number of days to 25% germination of *I. hilaris*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	40	342.88	<0.001
Light	1	40	105.97	<0.001
Priming:Light	4	40	42.79	<0.001

Table A.3.3. ANOVA table for the effects of aqueous smoke solutions on the number of days to 25% germination of *H. nudifolium*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	36	3817.31	<0.001
Light	1	36	83.21	<0.001
Priming:Light	4	36	31.28	<0.001

Table A.3.4. ANOVA table for the effects of aqueous smoke solutions on the number of days to 10% germination of *C. pospischilii*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	40	60714.12	<0.001
Light	1	40	112093.30	<0.001
Priming:Light	4	40	34086.67	<0.001

Table A.3.5. ANOVA table for the effects of aqueous smoke solutions on the number of days to 10% germination of *M. nerviglumis*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	36	3396.08	<0.001
Light	1	36	24166.30	<0.001
Priming:Light	4	36	4596.98	<0.001

Table A.3.6. ANOVA table for the effects of aqueous smoke solutions on the number of days to 10% germination of *H. capensis*.

Source of variation	numDF	denDF	F-value	P-value
Priming	4	36	8918.43	<0.001
Light	1	36	1626.92	<0.001
Priming:Light	4	36	5087.68	<0.001

Table A.3.7. ANOVA table for the effects of aqueous smoke solutions on the number of days to 25% germination of *S. arvensis*.

Source of variation	numDF	denDF	F-value	P-value
Light	1	91.77	18.06	<0.001
Priming	4	117.23	14.68	<0.001
Type	1	117.21	0.01	0.9430
Light:Priming	4	117.23	12.29	<0.001
Light:Type	1	92.24	8.20	0.0052
Priming:Type	4	117.23	17.39	<0.001
Light:Priming:Type	4	117.23	7.40	<0.001

B. Chapter 4 Supplementary Tables and Figures

Table B.1. ANOVA table for the effects of FPX-66 resin separated aqueous smoke solutions on germination percentage of *L. sativa* studied in the laboratory.

Source of variation	numDF	denDF	F-value	P-value
Resin fractions	2	144	35.91	<0.001
Dilutions (D)	6	144	85.19	<0.001
Resin fractions:D	8	144	10.08	<0.001

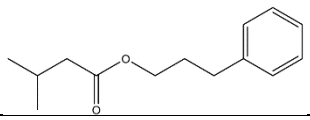
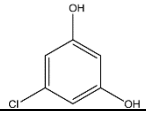
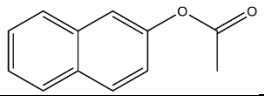
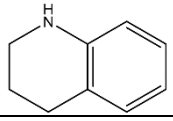
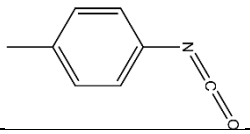
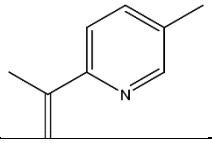
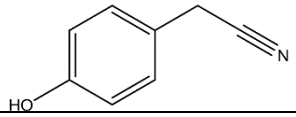
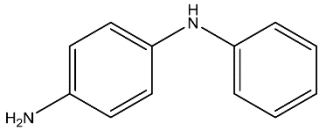
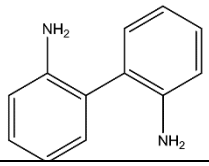
Table B.2. ANOVA table for the effects of liquid-liquid layer partitioned fractions (aqueous, organic, acidic and neutral) of aqueous smoke solutions on germination percentage of *L. sativa* studied in the laboratory.

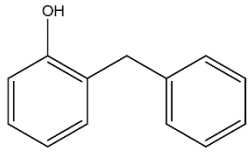
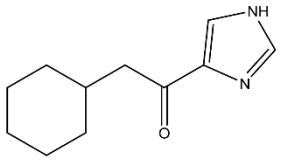
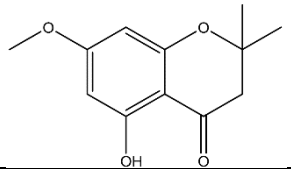
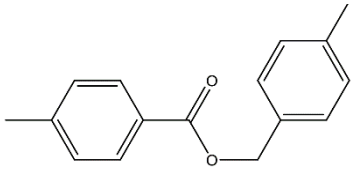
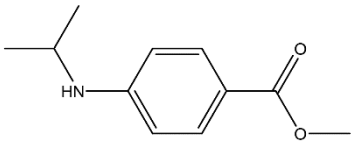
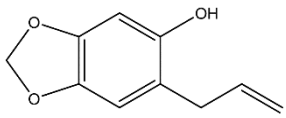
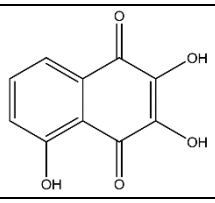
Source of variation	numDF	denDF	F-value	P-value
Partitioned fractions	3	198	70.13	<0.001
Dilutions (D)	6	198	358.02	<0.001
Partitioned fractions:D	13	198	21.90	<0.001

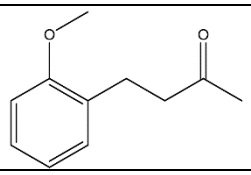
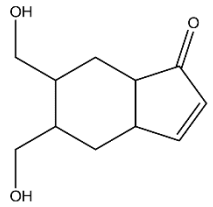
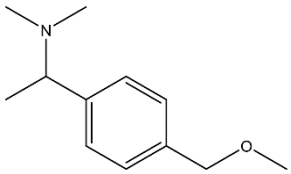
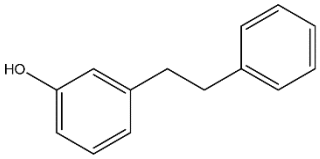
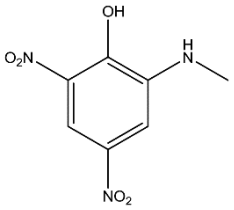
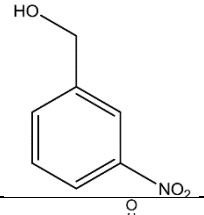
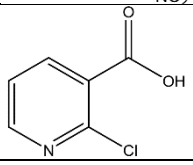
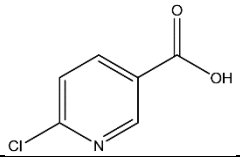
Table B.3. ANOVA table for the effects of tC18 fractions (n=6) of neutral smoke solutions on germination percentage of *L. sativa* studied in the laboratory.

Source of variation	numDF	denDF	F-value	P-value
C18 fractions	5	253.94	5.81	<0.001
Dilutions (D)	5	254.00	152.47	<0.001
C18 fractions:D	24	254.00	4.20	<0.001

Table B.4. Chemical compounds identified using GC-MS fragmentation patterns in conjugation with the NIST Mass library search (matching score > 400) in EtOAc neutral extract of alfalfa smoke solution.

Name	Molecular weight (g/mol)	Structure	Retention time (min)
3-phenylpropyl 3-methylbutanoate	220.14		11.55
5-chlorobenzene-1,3-diol	143.99		12.56
naphthalen-2-yl acetate	186.06		12.56
1,2,3,4-tetrahydroquinoline	133.08		15.1
1-isocyanato-4-methylbenzene	133.05		15.1
5-methyl-2-(prop-1-en-2-yl) pyridine	133.08		15.1
2-(4-hydroxyphenyl) acetonitrile	133.05		15.1
N1-phenylbenzene-1,4-diamine	184.10		22.94
biphenyl-2,2'-diamine	184.10		22.94

2-benzylphenol	184.08		22.94
2-cyclohexyl-1-(1H-imidazol-4-yl) ethanone	192.12		36.17
5-hydroxy-7-methoxy-2,2-dimethylchroman-4-one	222.08		36.17
4-methylbenzyl 4-methylbenzoate	240.11		39.77
methyl 4-(isopropylamino)benzoate	193.11		43.69
6-allylbenzo[d][1,3]dioxol-5-ol	178.06		43.69
2,3,5-trihydroxynaphthalene-1,4-dione	206.02		43.69

4-(2-methoxyphenyl) butan-2-one	178.09		45.39
5,6-bis(hydroxymethyl)-3a,4,5,6,7,7a-hexahydro-1H-inden-1-one	196.10		45.39
1-(4-(methoxymethyl)phenyl)- N, N-dimethylethanamine	193.14		45.39
3-phenethylphenol	198.10		45.39
2-(methylamino)-4,6-dinitrophenol	213.03		32.06
(3-nitrophenyl) methanol	153.04		32.06
2-chloronicotinic acid	156.99		23.98
6-chloronicotinic acid	156.99		23.98

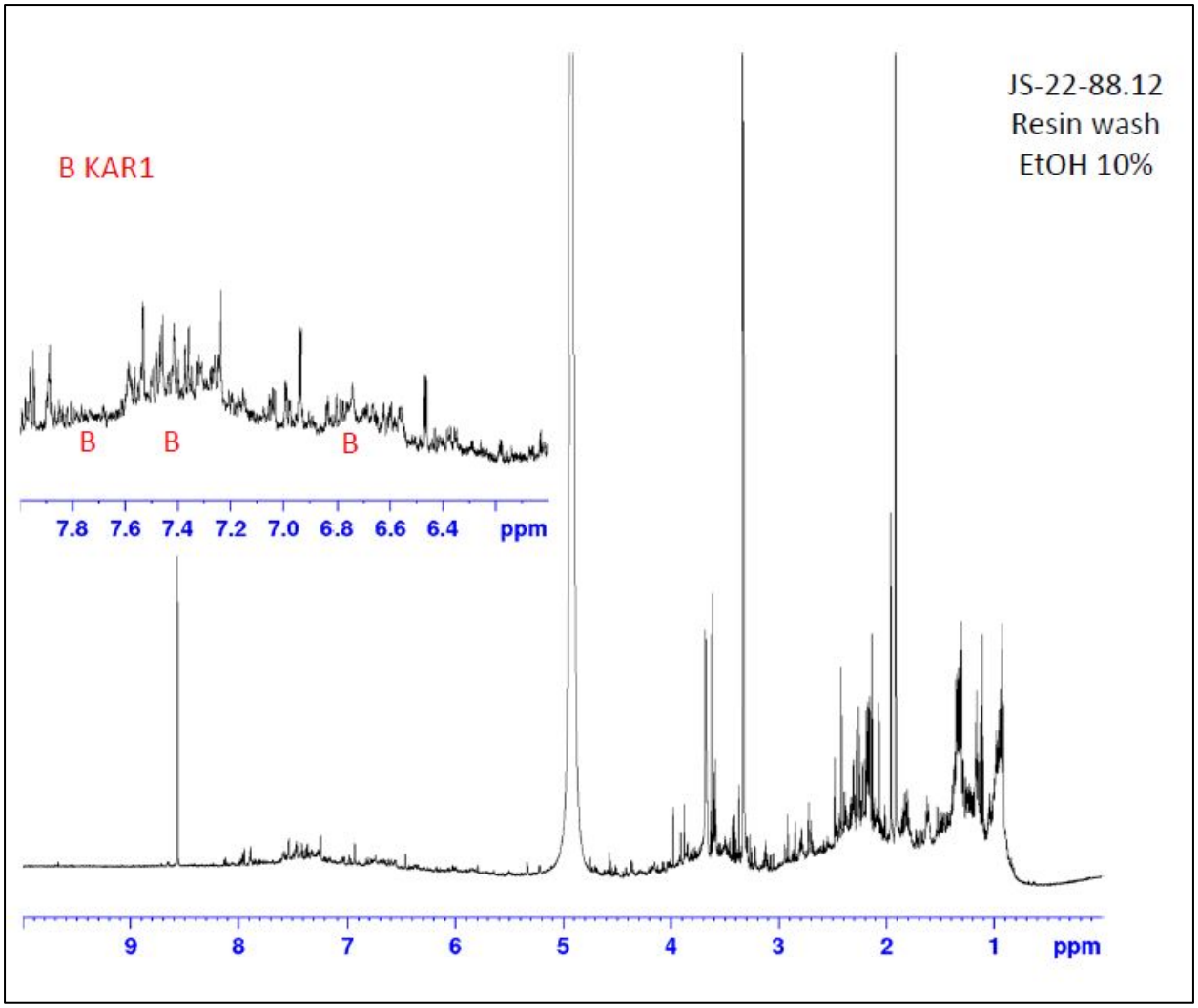


Figure B.1. 1D ^{1}H NMR spectrum of 10:90 ethanol: water desorbed fraction (in methanol-d_4 solvent) resulted after 1st desorption of FPX-66 resin with adsorbed alfalfa smoke compounds.

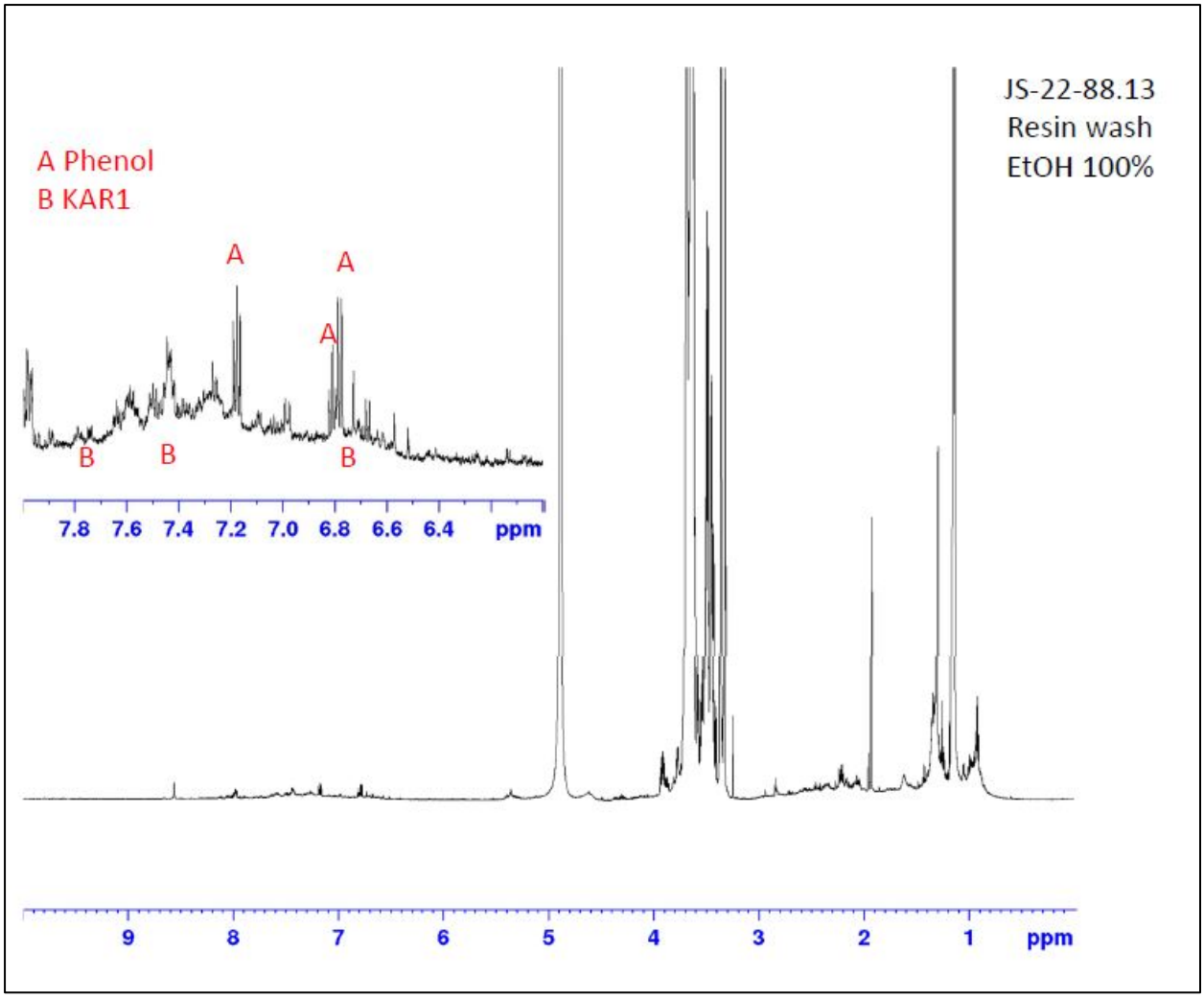


Figure B.2. 1D ^{1}H NMR spectrum of 100:0 ethanol: water desorbed fraction (in methanol-d_4 solvent) resulted after 2nd desorption of FPX-66 resin with adsorbed alfalfa smoke compounds.

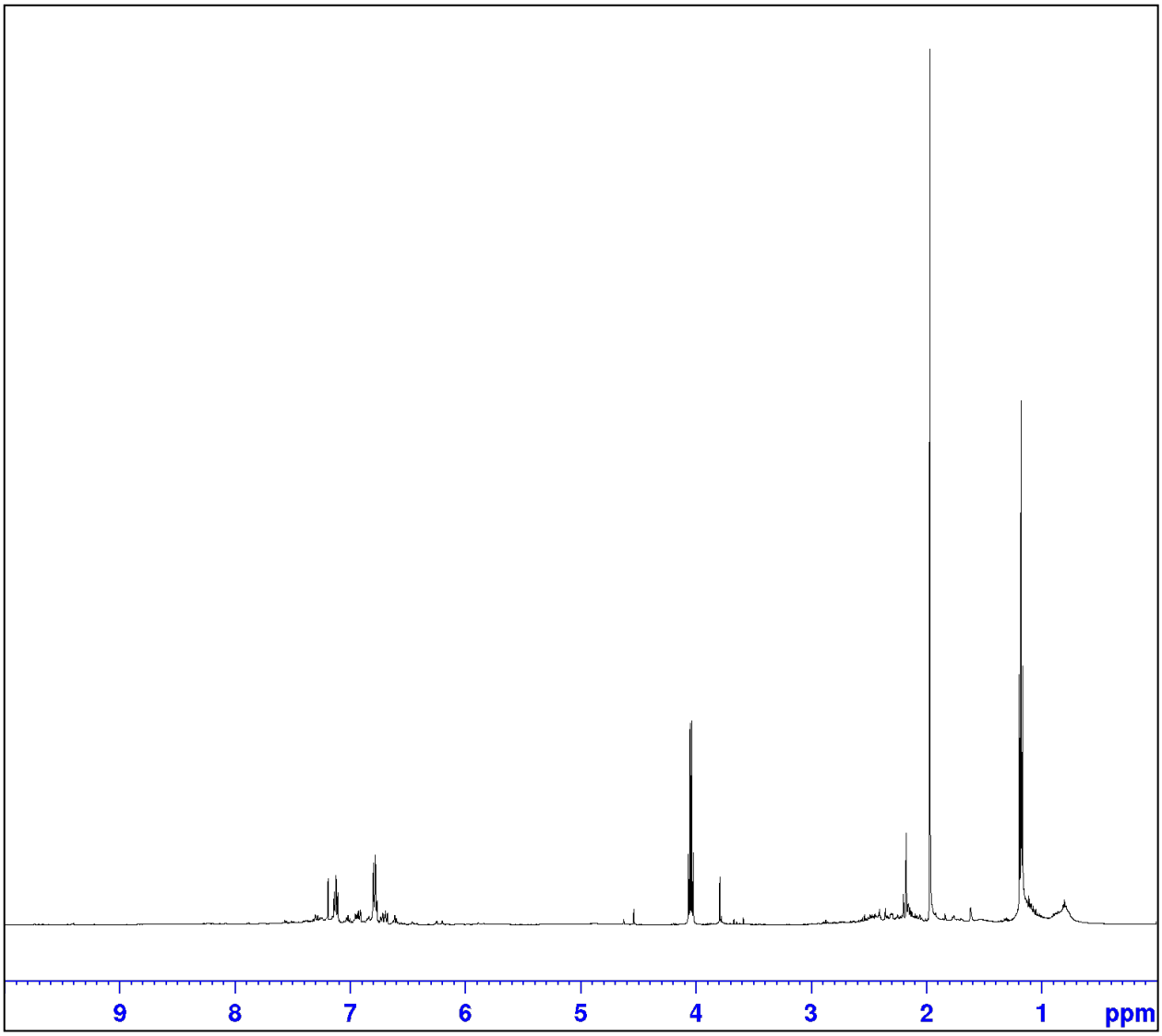


Figure B.3. ^{1}H NMR spectrum of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent.

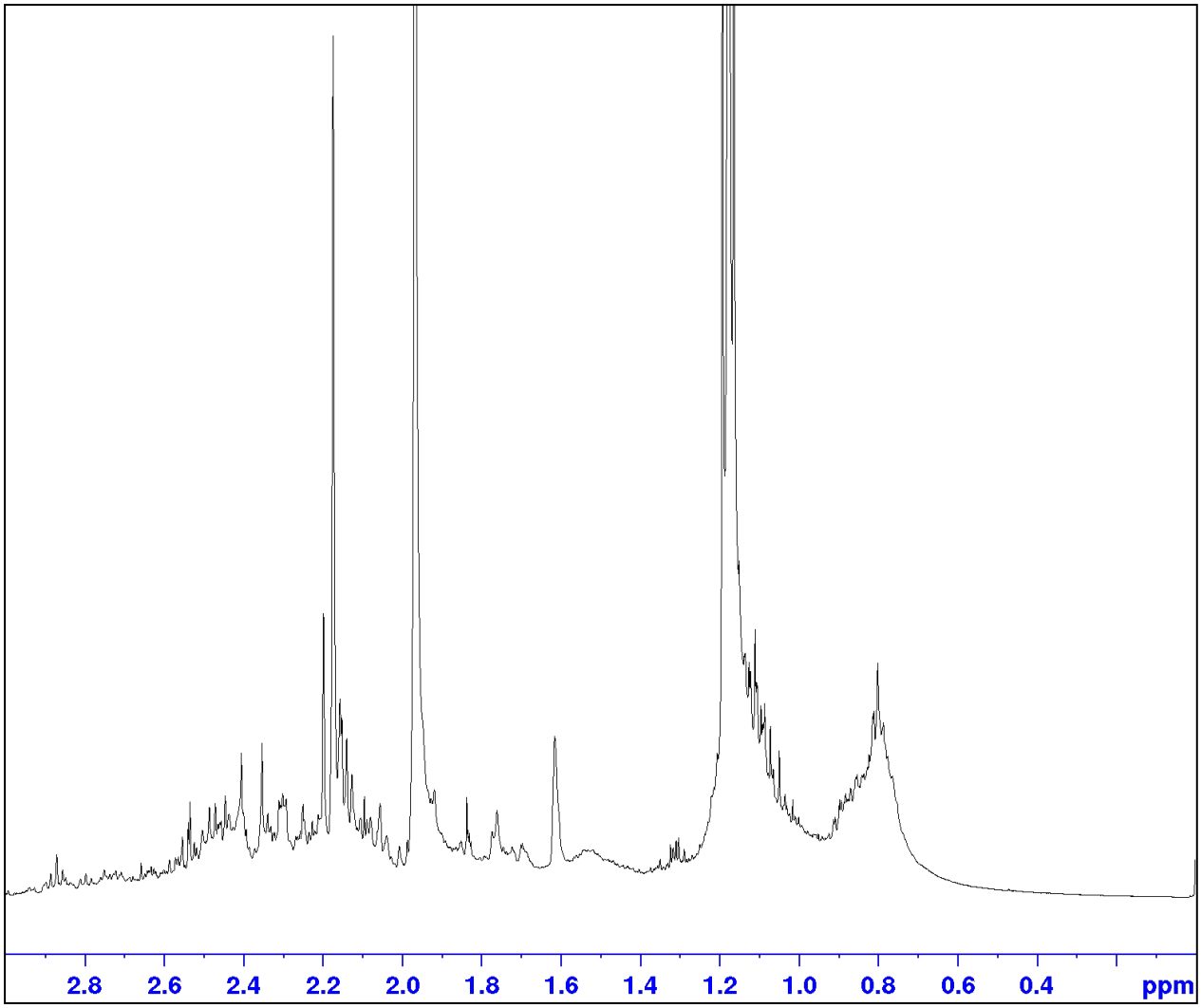


Figure B.4. ^{1}H NMR spectrum (0–3 ppm) of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: 3,4,5-trimethylfuran-2(5H)-one, Glyceronitrile, Acetic Acid, Acetic Acid methyl Ester, Propionic Acid, 1-Hydroxy-2-Butanone, 3-Furanol,Tetrahydro).

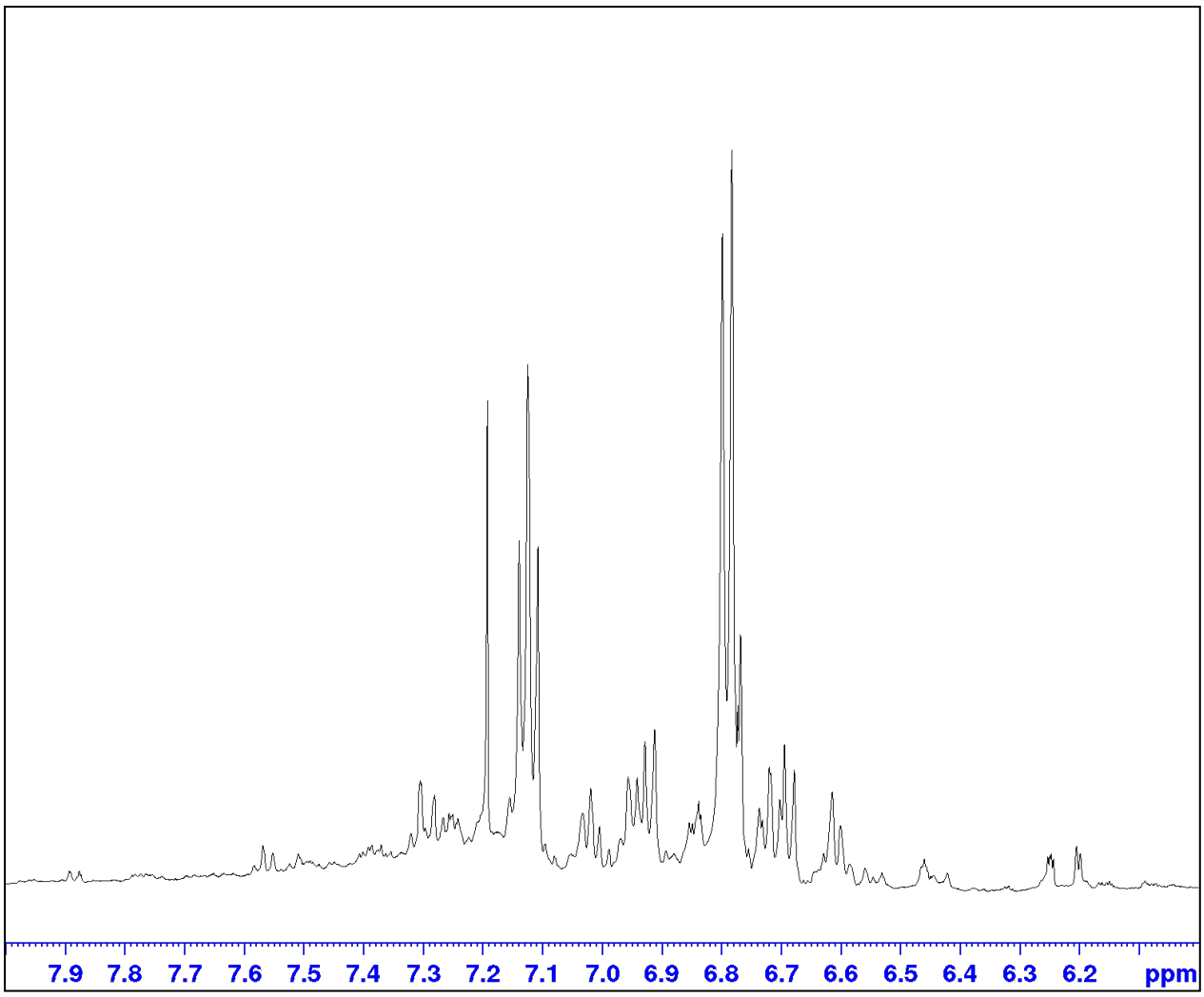


Figure B.5. ^{1}H NMR spectrum (6–8 ppm) of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: KAR1, 1,4-Benzenediol(hydroquinone), 1,2-Benzenediol(Catechol), 2-Furancarboxaldehyde(Furfural), 2,3,5-Trimethoxytouluene, Benzoic Acid, Phenol, Phenol,2-Methoxy, Phenol,3-Methoxy, Phenol,2,6-Dimethoxy, Phenol,3,4-dimethoxy, Phenol,3,6-Dimethoxy, 2-Methoxy-4-Methylphenol).

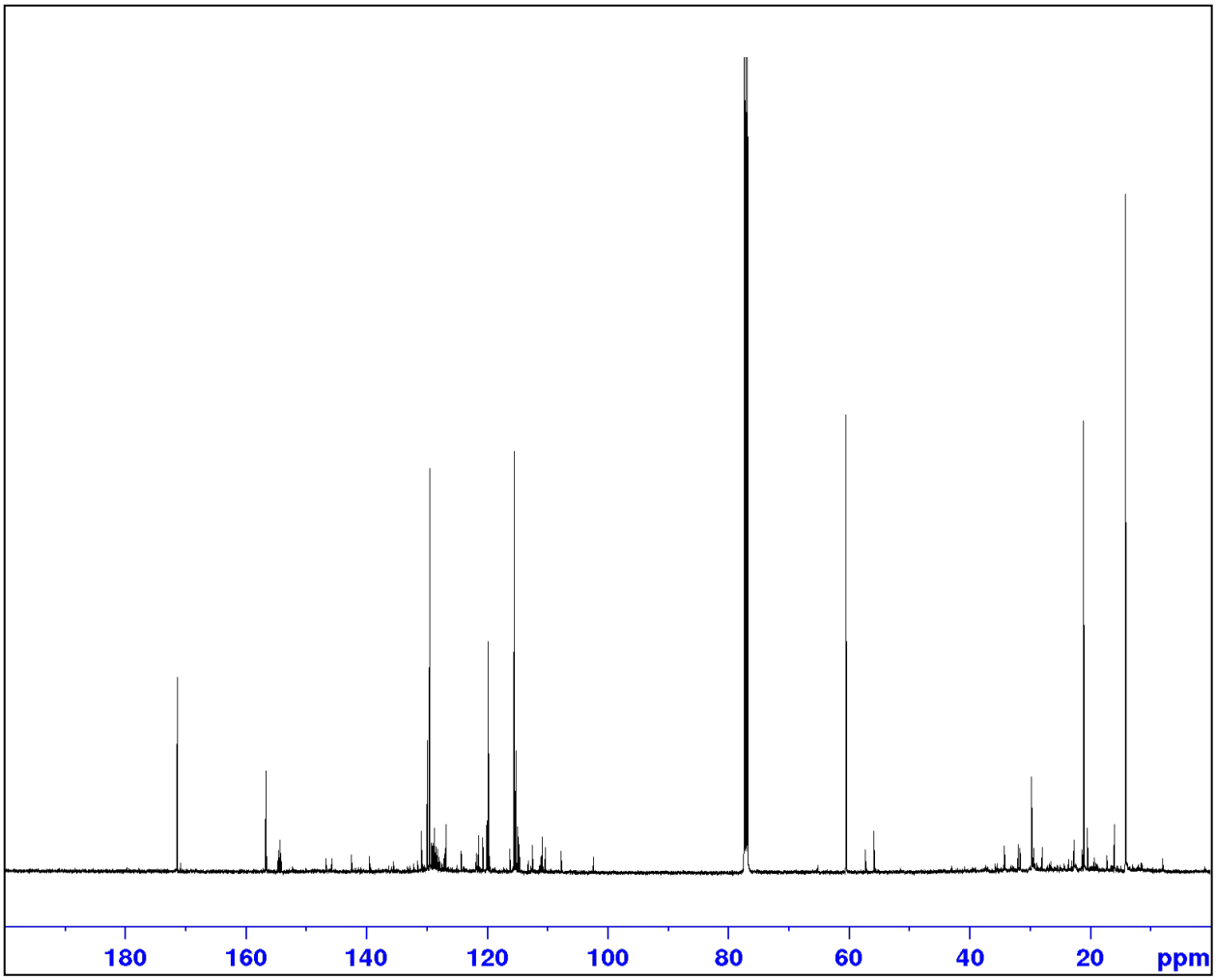


Figure B.6. ^{13}C NMR spectrum of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent.

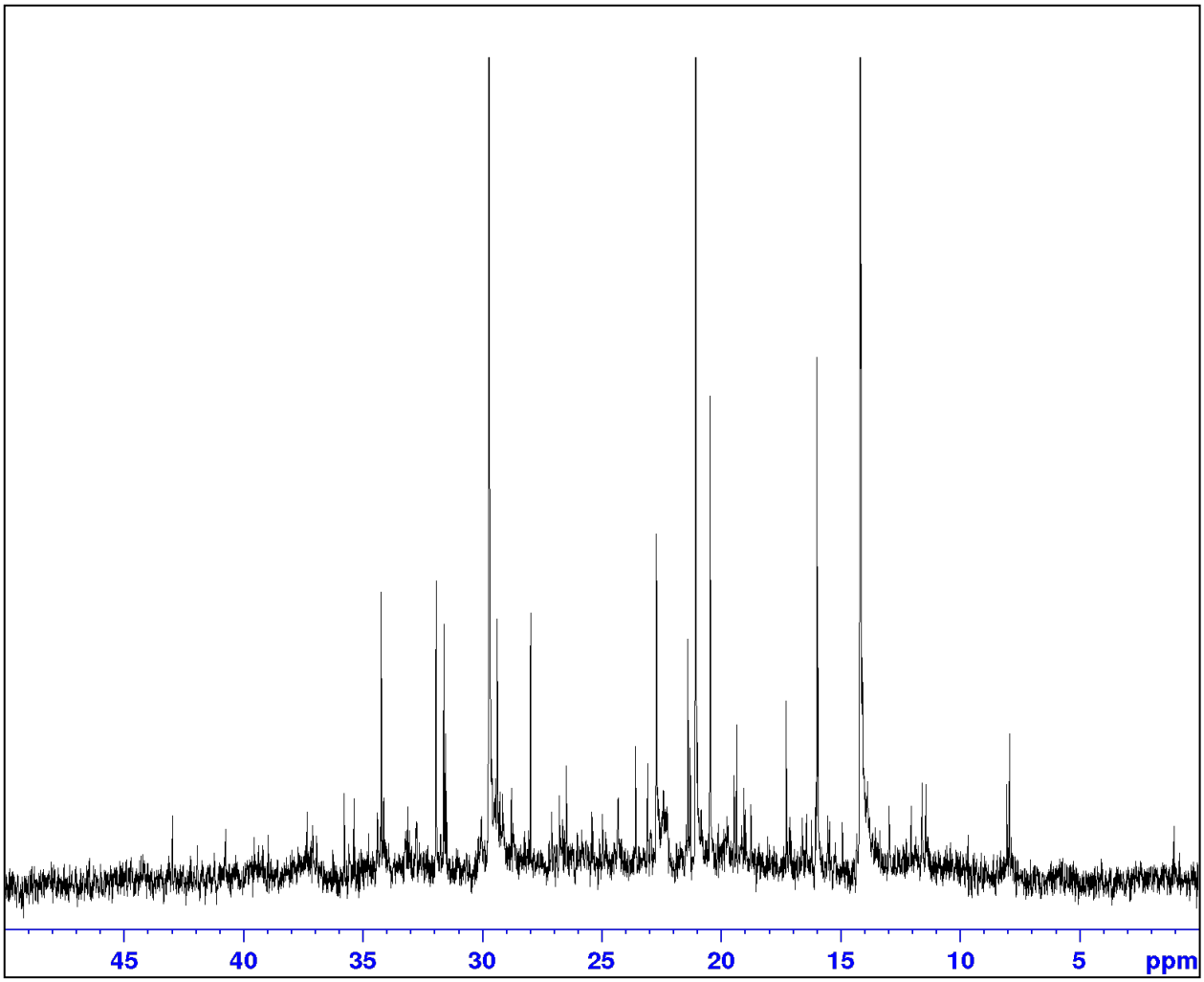


Figure B.7. ^{13}C NMR spectrum (0–50 ppm × 2) of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: 3,4,5-trimethylfuran-2(5H)-one, Glyceronitrile, Acetic Acid, Acetic Acid methyl Ester, Propionic Acid, 1-Hydroxy-2-Butanone, 3-Furanol,Tetrahydro).

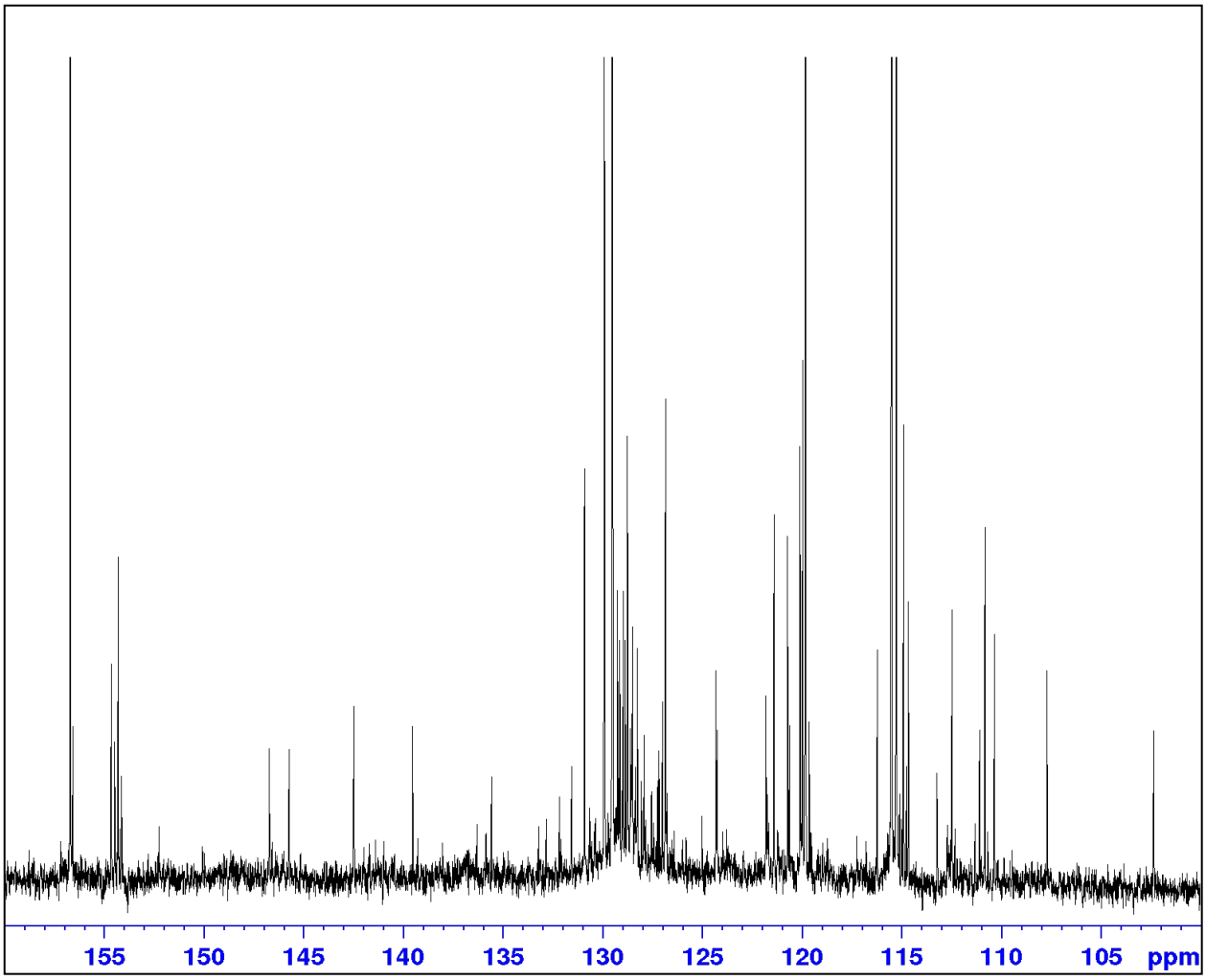


Figure B.8. ^{13}C NMR spectrum (100–160 ppm × 2) of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: KAR1, 1,4-Benzenediol(hydroquinone), 1,2-Benzenediol (Catechol), 2-Furancarboxaldehyde(Furfural), 2,3,5-Trimethoxytouluene, Benzoic Acid, Phenol, Phenol,2-Methoxy, Phenol,3-Methoxy, Phenol,2,6-Dimethoxy, Phenol,3,4-dimethoxy, Phenol,3,6-Dimethoxy, 2-Methoxy-4-Methylphenol).

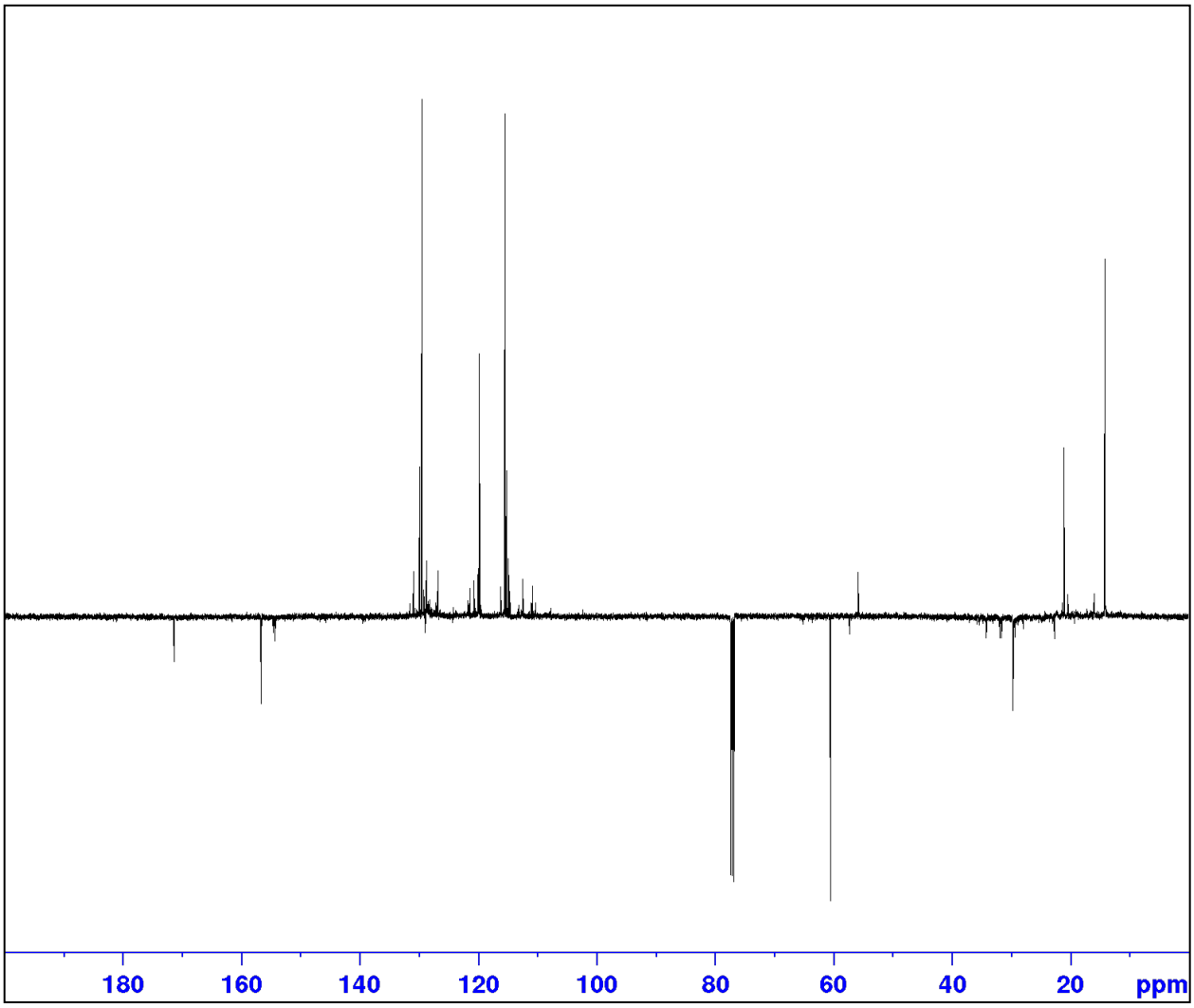


Figure B.9. APT NMR spectrum of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent.

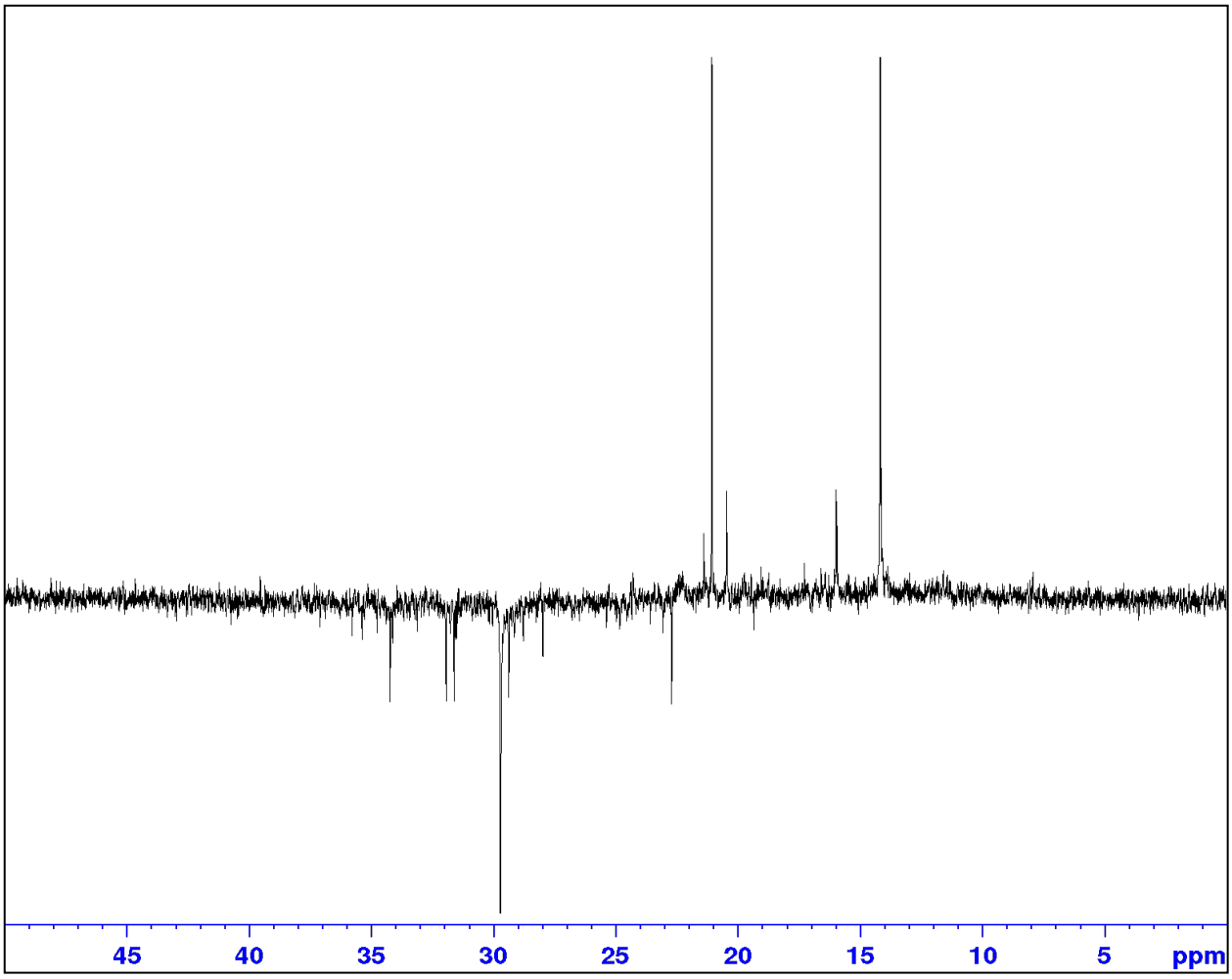


Figure B.10. APT NMR spectrum (100–160 ppm) of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: 3,4,5-trimethylfuran-2(5H)-one, Glyceronitrile, Acetic Acid, Acetic Acid methyl Ester, Propionic Acid, 1-Hydroxy-2-Butanone, 3-Furanol,Tetrahydro).

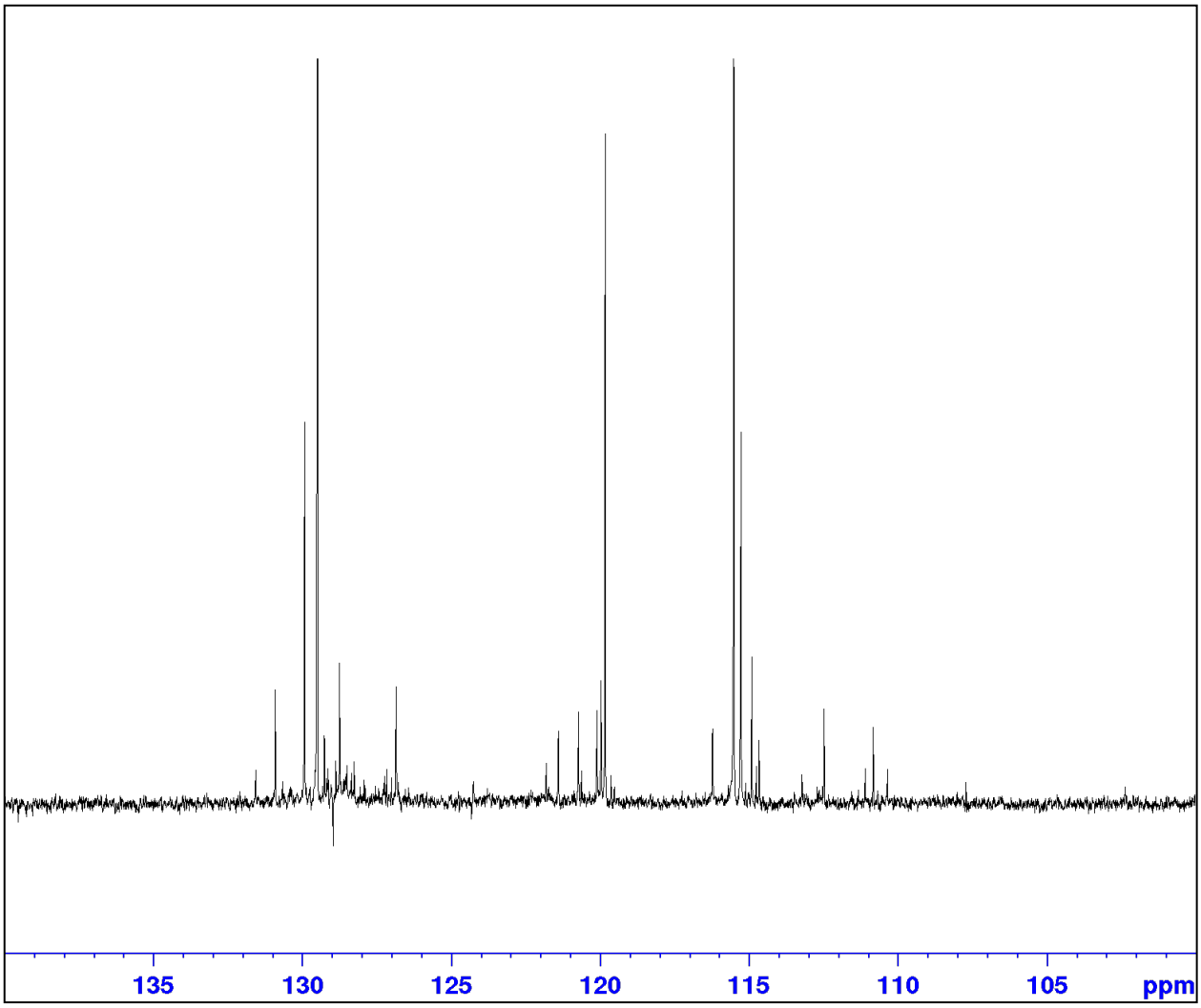


Figure B.11. APT NMR spectrum (100–140 ppm) of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: KAR1, 1,4-Benzenediol(hydroquinone), 1,2-Benzenediol(Catechol), 2-Furancarboxaldehyde(Furfural), 2,3,5-Trimethoxytouluene, Benzoic Acid, Phenol, Phenol,2-Methoxy, Phenol,3-Methoxy, Phenol,2,6-Dimethoxy, Phenol,3,4-dimethoxy, Phenol,3,6-Dimethoxy, 2-Methoxy-4-Methylphenol).

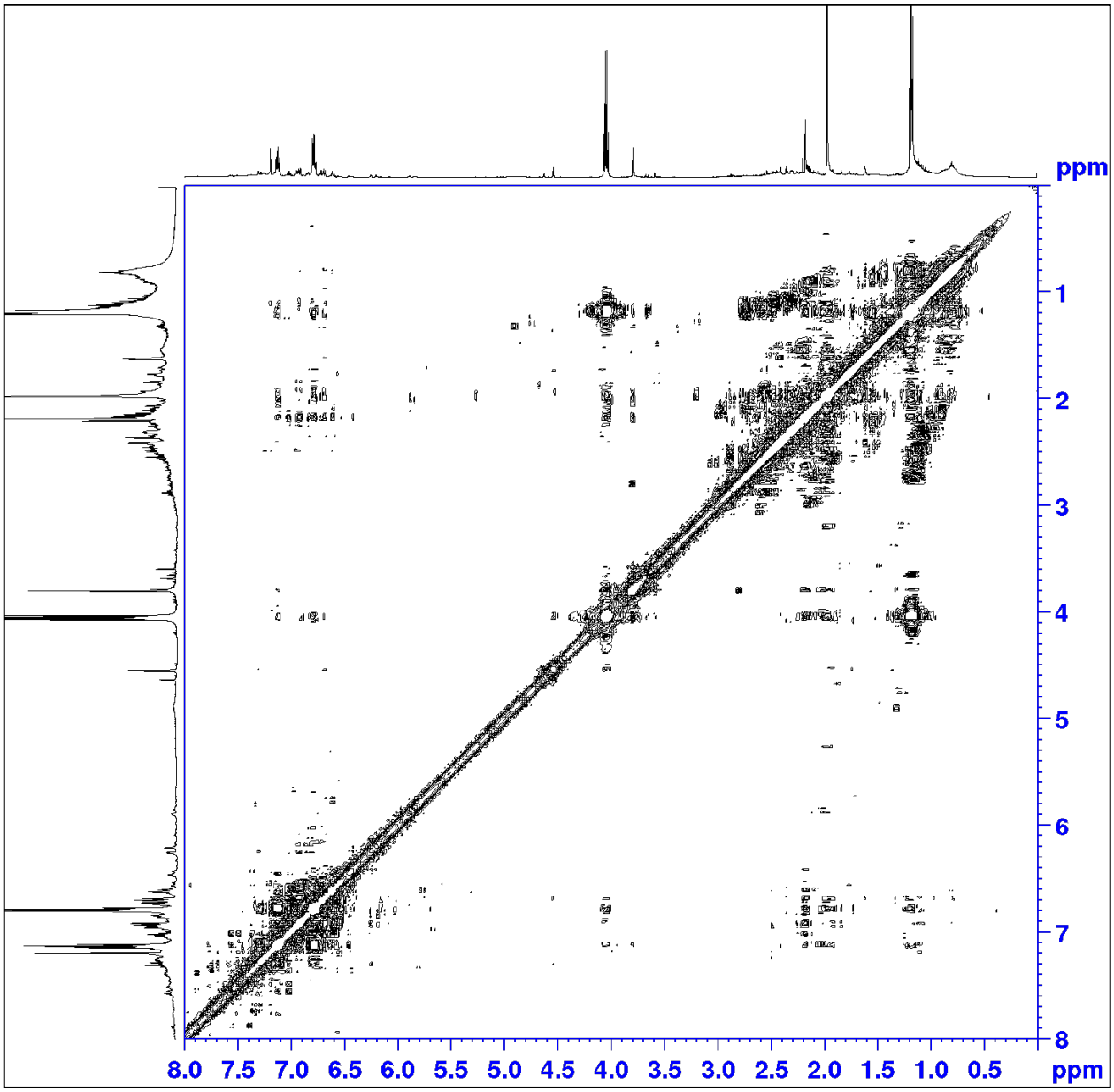


Figure B.12. 2D COSY (Correlation spectroscopy) NMR spectrum of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent.

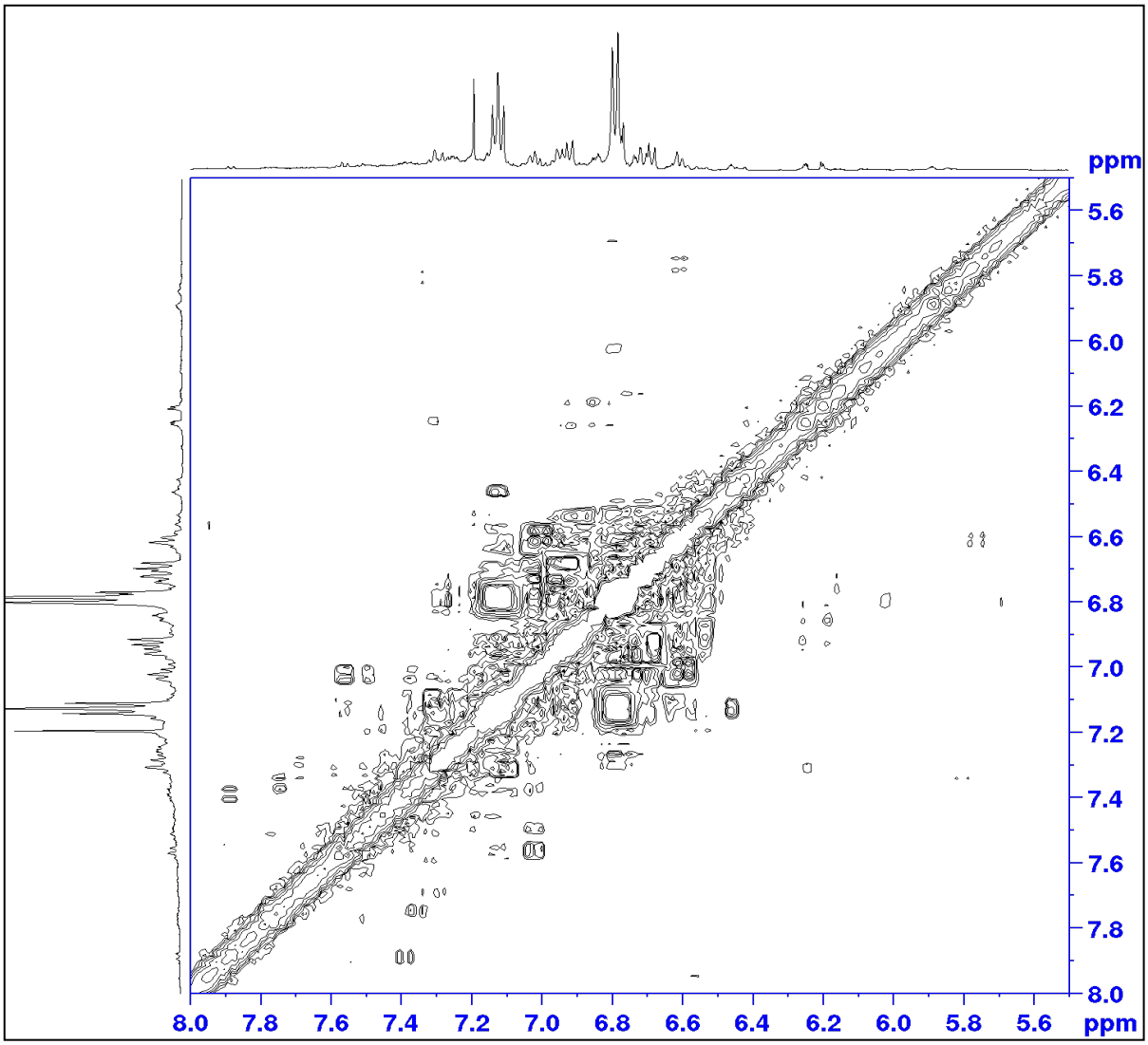


Figure B.13. 2D COSY (Correlation spectroscopy) NMR spectrum of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent (possible compounds from NMR simulation: KAR1, 1,4-Benzenediol(hydroquinone), 1,2-Benzenediol (Catechol), 2-Furancarboxaldehyde(Furfural), 2,3,5-Trimethoxytouluene, Benzoic Acid, Phenol, Phenol,2-Methoxy, Phenol,3-Methoxy, Phenol,2,6-Dimethoxy, Phenol,3,4-dimethoxy, Phenol,3,6-Dimethoxy, 2-Methoxy-4-Methylphenol).

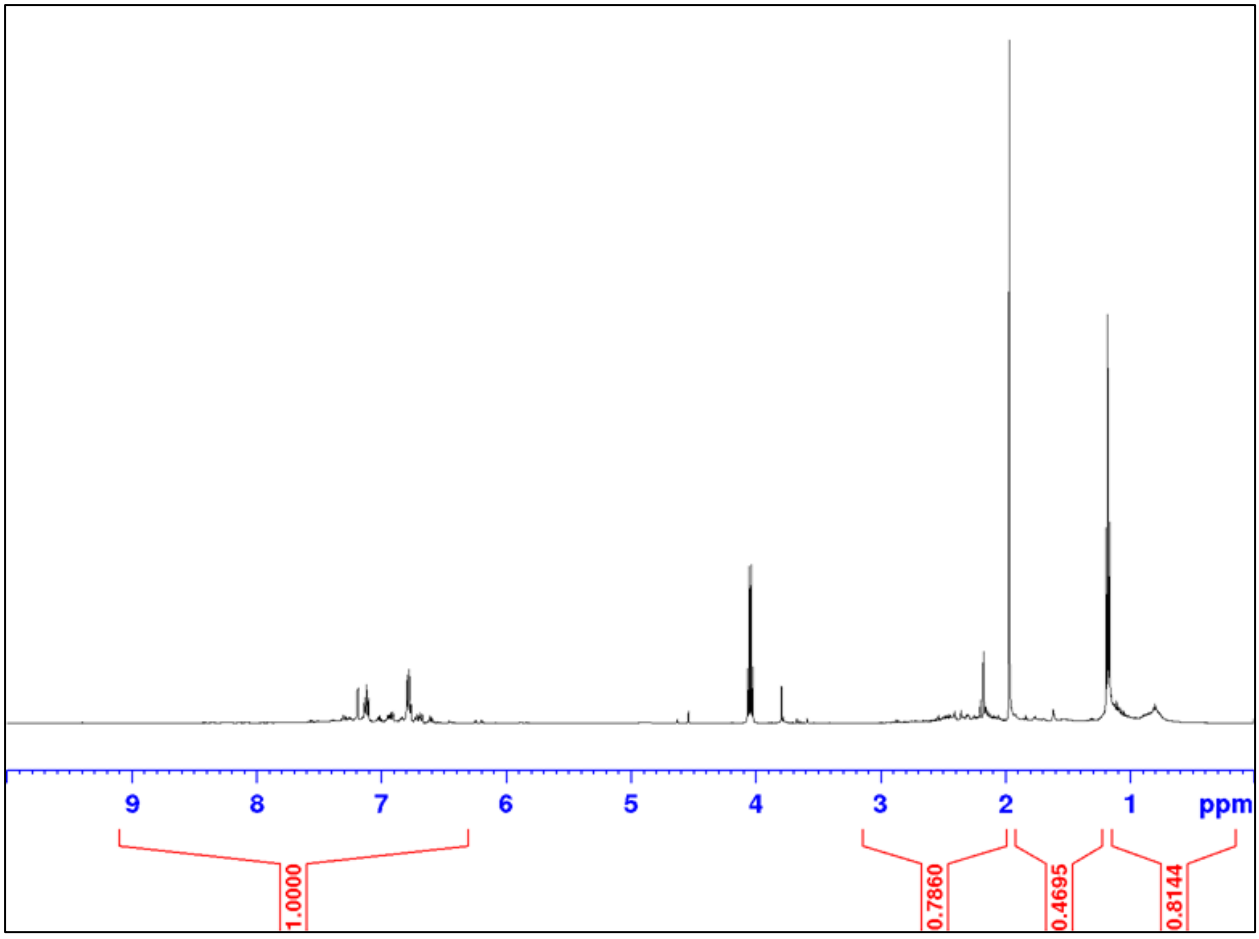


Figure B.14. ^{1}H NMR spectrum with integrated values for aromatic quantity determination of EtOAc extracted neutral fraction of alfalfa smoke solution in $CDCl_3$ solvent.